COMPARATIVE IMMUNOLOGICAL DEVELOPMENT AND RESPONSES IN LOWER VERTEBRATES: STURGEON

BY

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ABSTRACT

Sturgeon are harvested worldwide for their meat and eggs, sold as caviar and are among the most economically important fish species worldwide. A generally low physiological response to external stimuli/stressors has been documented in these phylogenetically primitive species.

Considering how little is known about sturgeon immune responses, it is important to study how they respond to such stimuli/stressors. The culture of sturgeon larvae is associated with a high mortality rate during and after yolk sac absorption and onset of feeding. Therefore, it is relevant to study the immune competence of larvae and juvenile sturgeon and determine when their immune organs first appear and how they develop. The meningeal myeloid tissue, the spleen and the thymus were characterized morphologically by light microscopy (LM) and transmission electron microscopy (TEM) in Atlantic sturgeon (Acipenser oxyrinchus oxyrinchus) during the first 5 months of life (until 2895 growing degree days, °C.day (dd)). In sturgeon, the spleen was first visible approximately 400 dd after the onset of feeding (at 541 dd) and the meningeal myeloid tissue and the thymus at 768 dd after the onset of feeding; these first appeared between 541 dd and 768 dd, respectively. Heterophil and eosinophil percentages were significantly greater in the meningeal myeloid tissue of larger fish (950 and 2895 dd, respectively) when compared with the smaller fish. The percentages of reticular cells were significantly higher in the smallest fish than in the larger fish and the undifferentiated cells were higher in the youngest animals compared to the older age groups. Likewise, the splenic heterophil percentages were significantly higher in the oldest fish (2895 dd) compared to the youngest fish (768 dd). The splenic undifferentiated cell percentages were significantly higher in the younger fish compared

to the oldest. This emphasizes less developed immune organs in the smaller animals. Lymphocyte percentages did not change over time in these tissues. In the thymus, lymphocytes were the predominant cell type and only the undifferentiated cell percentages were significantly different and highest in the oldest group, possibly reflecting a more proliferative thymus in these animals.

In order to characterize changes in cell populations in relevant immune organs, a temperature trial was done in juvenile shortnose sturgeon (Acipenser brevirostrum), kept at 11°C or at 20°C. Relevant immune organs such as the meningeal myeloid tissue, the spleen, the thymus and the skin were studied. A higher temperature had a significant positive influence on the immune cell production in the meningeal myeloid tissue (higher lymphocyte, eosinophil and heterophil percentages). Likewise, temperature resulted in an initial but transient increase in splenic white pulp percentage. A similar effect was seen for splenic expression of interferon regulatory factors 1 and 2 (IRF-1 and IRF-2) and no changes occurred in matrix metalloproteinase 9 (MMP-9) expression for both spleen and skin. IRF-1 and IRF-2 expression in the skin were significantly higher expression in the fish kept at 11°C at 3 and 6 weeks post temperature change. There were no differences in thymus size between the different groups. Further research is required to better characterize the nature of these immune responses in these organs.

These findings suggest that an effective immune response may not occur in early life stages prior and during the onset of feeding but further research is needed to further assess this. Rearing sturgeon juveniles at higher temperatures (up to 20°C) may be beneficial for sturgeon aquaculture, since it maximizes growth rates and positively influences immune cell percentages in relevant immune organs.

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LIST OF ABBREVIATIONS

AP	**Alkaline phosphatase**
APCs	Antigen presenting cells
Bdw	Body weight
CITES	Convention on International Trade in Endangered Species of Wild Fauna and Flora
CTGF	Connective tissue growth factor
CTLs	Cytotoxic T lymphocytes
dd	Growing degree days
dpf	Day post fertilization
dph	Day post hatch
EF-1	Elongation factor 1
ELISA	Enzyme-linked immunosorbent assay
hpf	Hour post fertilization
H&E	Hematoxylin and eosin stain
IFN	Interferon
IFNs	Interferons
Ig	Immunoglobulin
Igs	Immunoglobulins
IL	Interleukin
IPNV	Infectious pancreatic necrosis virus
IRF	Interferon regulatory factor
IRFs	Interferon regulatory factors
LM	Light microscopy
LPS	Lipopolysaccharides
MAC	Membrane complex attack
MHC	Major Histocompatibility Complex
MMC	Melanomacrophage centers
MMP	Matrix metalloprotease
MNRQ	Mean Normalized Relative Quantity
MPO	Myeloperoxidase

NBF	Neutral buffered formalin (10%)
NK cells	Natural killer cells
nm	Nanometers
PAMPs	Pathogen associated molecular patterns
PCV	Packed cell volume
PCVs	Packed cell volumes
PO	Propylene oxide
Poly I:C	Poly inosinic:cytidylic acid
ppm	Parts per million
PRRs	Pattern recognition receptors
qPCR	Real time quantitative polymerase chain reaction
RAG	Recombination-activating gene
RINs	RNA integrity numbers
RT	Room temperature
SE	Standard error bars
SEM	Standard Error of the Mean
TCRs	T-cell receptors
TEM	Transmission electron microscopy
TGF-β	Tumor growth factor-β
TLRs	Toll-like receptors
TMS	Tricaine methanesulfonate
TNF- α	Tumor necrosis factor- α
TRI Reagent	Trizol Reagent
Wpf	Week post fertilization
WSAV	White sturgeon (Acipenser transmontanus) adenovirus
WSHV	White sturgeon herpesvirus
WSIV	White sturgeon iridovirus
µl	Microliter
µm	Microns

Chapter 1.0 General Introduction

1.1. Sturgeon phylogeny

Jawed fishes (Gnathostomata) arose around 400 million years ago. Their descendants evolved into two major groups, the Chondrichthyes (cartilaginous fish, the most primitive of extant jawed vertebrates) and the Osteichthyes (bony fish). Cartilaginous fish include the Elasmobranchii (sharks, skates, rays) and the Holocephali (chimeras). Bony fish are currently the most diverse group in salt and freshwater systems and include the Teleostei (modern fish) and the Chondrostei (sturgeon and paddlefish)[1]. Chondrostei form a transition between major taxa and are therefore relevant for the understanding of vertebrate evolution[2]. Within the Chondrostei, the order Acipenseriformes comprises two families: Polydontidae (one genus with two paddlefish species) and Acipenseridae (five genera with twenty five sturgeon species)[3].

1.2. Sturgeon biology

Sturgeon aquaculture has increased considerably in the last two decades, with the world production of farmed caviar for all species in 2008 estimated in the order of 110–120 tons, from around 80 farms in 16 countries. A total of 35 countries are presently involved in sturgeon aquaculture for meat and caviar[4]. Excessively harvested for their meat and eggs, sturgeon are threatened or endangered species listed under the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) in Appendices I and II (http://www.cites.org/eng/resources/species.html).

Sturgeon derive from a common ancestor of teleosts over 200 million years ago and possess a cartilaginous skeleton and primitive features such as a heterocercal tail fin,

notochord and protective rows of scutes. These species may migrate between marine and freshwater systems (diadromous) or within freshwater systems (potamodromous), all spawning in fresh water. Their longevity, late sexual maturation and migration patterns make them very susceptible to excessive harvesting and habitat degradation. There are 27 extant chondrostean species[3] and this dissertation focuses on the immunological development of 2 of the North American sturgeon species, the Atlantic sturgeon (Acipenser oxyrinchus oxyrinchus) and the shortnose sturgeon (Acipenser brevirostrum).

1.3. Sturgeon life history

1.3.1. Atlantic sturgeon distribution

Atlantic sturgeon have been listed in CITES Appendix II since 1979, meaning that trade must be non detrimental to its survival in the wild (http://www.cites.org/eng/resources/species.html). Atlantic sturgeon are anadromous and live most of their adult life at sea, migrating to freshwater systems to spawn[5]. Atlantic sturgeon are represented by two subspecies: A. oxyrinchus oxyrinchus and A. o. desotoi. The first subspecies' habitat ranges from Hamilton Inlet (coast of Labrador) to the St. Johns River in Florida; A. o. desotoi inhabits the Gulf of Mexico coast of Florida to northern South America[5]. Atlantic sturgeon are currently present in 35 rivers, spawning in at least 20 of them[6]. Marine habitat preferences are likely related to greater foraging opportunities[7].

Atlantic sturgeon reach an average age of 60 years and, like all sturgeon species, are late maturing. Sexual maturation varies with gender and latitude, occurring earlier in southern species. In the Saint Lawrence River, males mature at 22-34 years and females at 27-28 years. Spawning occurs in fresh water in the spring/early summer (May-June in

the Saint Lawrence River), close to the fresh and saltwater interface of spawning migrations and it seems to be mainly temperature regulated. Juvenile sturgeon migrate to brackish waters and reside in estuarine waters for months or years. Sub-adults (76-92 cm in total length, age between young-of-year and that of mature adults) may move to coastal waters and travel widely once they leave the rivers[6]. Despite the CITES listing of Atlantic sturgeon in Appendix II, there is a fishery in the Saint John River in New Brunswick with a quota of 350 fish per year. Many of the females collected are used for establishing a cultured stock from their progeny at the Acadian Sturgeon and Caviar Inc. hatchery in Carter's Point, New Brunswick (NB).

1.3.2. Shortnose sturgeon distribution

Shortnose sturgeon have been listed in CITES Appendix I since 1975, meaning that no commercial trade is permitted (http://www.cites.org/eng/resources/species.html). Shortnose sturgeon are amphidromous, moving between fresh and saltwater systems during their life cycle but not for breeding purposes, and are present most frequently in river systems[8]. There are 19 distinct population segments of shortnose sturgeon inhabiting 25 river systems ranging from the Saint John River, NB, to the St. Johns River, Florida[9]. Northern adults live 30 to 67 years and southern adults live 10 to 25 years. Adults reach a maximum length of 120 cm and approximately 24 kg, resembling similar sized juvenile Atlantic sturgeon[8]. Males first spawn at around 12 years of age and probably at 2 year intervals thereafter; females spawn at 18 years of age and at 3 to 5 year intervals thereafter. Spawning occurs in fresh water, riverine sections of the upper estuary during May-June at 10-15°C[10]. Shortnose sturgeon undergo earlier migration and spawning than Atlantic sturgeon at comparable latitudes. Temperature is likely the

major contributing factor in determining migration and spawning behaviors, given that spawning season occurs later at higher latitudes for both species[11]. Tolerance of early life stages to increasing salinity and low dissolved oxygen increases with age[12] with juveniles remaining in fresh water until they attain 45 cm before they join the regular annual migration of adults upstream in the spring-summer and towards the ocean in the fall[10].

1.4. Sturgeon physiology

To date, most of the research done on sturgeon has addressed conservation biology, ecology and aquaculture aspects of the most economically viable species. These include the Eurasian species beluga sturgeon (Huso huso), starry sturgeon (A. stellatus), Persian sturgeon (A. persicus) and Russian (diamond) sturgeon (A. gueldenstaedtii) as well as the North American white sturgeon (A. transmontanus) and Atlantic sturgeon (A. oxyrinchus oxyrinchus)[13]. Conservation programs rely on scientific knowledge but little is known in sturgeon about their physiology and response mechanisms towards environmental stimuli. A generally low physiological response to external stimuli or stressors has been documented in these phylogenetically primitive species. The pallid sturgeon (Scaphirhynchus albus) exhibited no significant changes in plasma cortisol, lactate and glucose concentrations when subjected to a 30 second aerial net stressor[14]. They also showed no cortisol responses when injected with lipopolysaccharide (LPS), unlike the responses seen in teleost species such as the yellow perch (Perca flavescens)[15]. A review of the literature available on Atlantic and shortnose sturgeon reveals little regarding their physiology and immune responses. Juvenile Atlantic and shortnose sturgeon had, when compared with teleosts, lower oxygen-carrying capacity,

lower concentrations of blood metabolites as well as a lower physiological response to forced activity, hypoxia and hypercapnia[16]. Given the endangered status of these species and their economic value it is important to learn more of how their immune responses develop in early life stages and how they respond to environmental stimuli or stressors such as water temperature changes and pathogens.

1.5. Fish immunology

1.5.1. Maternal influences and investment on progeny

Sturgeon species are long-lived[10]. The longevity of fish species may have beneficial effects on spreading larval production over variable environmental conditions and a long period of time[17]. Factors such as nutrition and stress levels of the mother significantly influence the offspring[18]. Females with a higher nutritional intake produce embryos with larger yolk sacs prior to hatching and with higher hatching success and longer survival after hatching[19]. Also, studies on feed-supplemented black rockfish (Sebastes melanops) showed that maternal age on larval performance was significant. Larvae from older females grew faster in both length and mass when compared with larvae from younger females[20]. In non-supplemented animals, larvae from older females survived starvation longer than larvae from younger females[20]. All these factors influence the survival of the fish offspring. Considering the longevity of sturgeon species, older females may have a significantly positive impact on the quality and survival rate of eggs and larvae, both in the wild and in aquaculture settings.

In vertebrates, the mother can also contribute to the survival of its progeny by transfer of immune factors via the egg[21,22]. The maternal transfer of non-specific immune factors has been documented in fish. These include lectins in rock bream

(Oplegnathus fasciatus) eggs[23] and lysozyme and complement in zebrafish (Danio rerio) egg cytosol[22]. Lysozyme in coho salmon (Oncorhynchus kisutch) eggs has been shown to play a role in the prevention of vertical transmission of some bacterial pathogens like Aeromonas salmonicida[24]. Vitellogenin (the major protein of yolk) is another component with immune properties and has documented hemagglutinating and bacteriostatic activities in amphioxus (Branchiostoma belcheri tsingtauense)[25] and in the teleost rosy barb (Puntius conchonius)[26]; it may therefore contribute to larval defense mechanisms. Vitellogenin has also exhibited potent antiviral activity and a strong neutralizing effect against the salmonid infectious pancreatic necrosis virus (IPNV)[27]. This antiviral activity may occur in other fish species such as sturgeon.

Maternal transfer of specific immune factors such as IgM has been documented in several species such as the Atlantic salmon (Salmo salar), rainbow trout (O. mykiss) and Indian major carp (Labeo rohita)[28]. A positive correlation was found between the level of immunoglobulins (Igs) in plasma of mature Atlantic salmon females and the Ig content in their eggs. The immunoglobulin (Ig) levels in the eggs declined gradually from the day of fertilization until very low levels were present by onset of the first feeding at day 130 after fertilization. Thereafter, Ig levels in the fry homogenates increased rapidly, probably due to autologous synthesis[29]. Maternal transfer of Igs may compensate for the relative late appearance of autologous humoral IgM. Also, immunity appears to be mostly a function of size rather than age. Maximum protective immunity in fry of several salmonid species against pathogens such as Yersinia ruckeri and Vibrio anguillarum occurred when they reached a minimum size of 1 to 2.5g, with differences in response indicated among several species[30].

1.5.2. Lymphoid organs in fish

In lower vertebrates, lymphoid tissue is usually associated with myeloid/hemopoietic tissue, forming lymphomyeloid organs similar to bone marrow. Elasmobranchs have well developed and characteristic lymphomyeloid organs such as the Leydig's organ and epigonal organ and in holocephalans (Chimaera), lymphomyeloid tissue is found in the cranium. In all fish except for cyclostomes, the kidney and spleen are important lymphomyeloid organs and the thymus is an important lymphoid organ[31]. In the holocephalan Chimaera monstrosa, the spleen, meningeal lymphoid tissue and the thymus have been previously described and characterized[32]. In sturgeon, the main lymphomyeloid tissues are the anterior part of the kidney, the meningeal tissue, the spleen, the thymus, the pericardial tissue and lymphoid masses of the intestine, especially in the spiral valve[33,34]. In sturgeon, the hemopoietic tissue arises from derivatives of the coelomic wall (spleen, epicardium and serosa of the intestines) and of skeletogenic tissue, which is an evolutionarily more recent tissue of chordates[34]. In sturgeon, the spleen serves as a blood reservoir and is involved in lymphopoiesis. As in higher vertebrates, the sturgeon spleen is differentiated into white and red pulp and has large arteries surrounded by large lymphoid follicles. The thymus is lobulated, divided into cortex and medulla and contains lymphocytes, reticular cells and macrophages, but no Hassall's corpuscles[33]. The similarity between the morphological structure of the sturgeon thymus and the thymus of higher vertebrates suggests that they are functionally similar[34]. In teleosts as in most vertebrates, the thymus involutes with age. The relative weight of the thymus (percentage of body weight) decreases dramatically in rainbow trout at two months of age, remaining constant afterwards[35]. The thymus also showed signs of involution in adult zebrafish (15 weeks post

fertilization (wpf)) with atrophy, decreased density of lymphocytes in the cortex along the pharyngeal epithelium and subsequent replacement by connective tissue and epithelial cells[36]. However, in phylogenetically primitive fish such as the holocephalan C. monstrosa and the sterlet (Acipenser ruthenus), the thymus does not undergo complete involution with age[32].

The pericardial and the meningeal myeloid tissues are hemopoietic sites in sturgeon. The pericardial tissue is predominantly lymphoid subdivided into smaller compartments separated from one another by connective tissue, each filled with lymphocytes, reticular cells, granulocytes and scattered macrophages.[33] The meningeal myeloid tissue is found within the cranial cavity above the hind part of the brain and the anterior part of the spinal cord. It is lobulated with irregular blood or lymph-filled sinuses and the lobes contain loosely packed free cells interspersed with connective tissue, small arteries and nerve bundles[33].

1.5.3. Innate immune system

In all multicellular organisms, the immune system evolved due to the need to cope with pathogens and infectious microorganisms. As a result, several defense mechanisms have evolved and been conserved across different plants, invertebrates and vertebrates:

Pattern recognition receptors: The innate system is a phylogenetically ancient component of the immune response and is based on germline-encoded receptors for the recognition of conserved molecular patterns characteristic of microbial pathogens, collectively called pathogen-associated molecular patterns (PAMPs)[37]. These receptors are termed pattern recognition receptors (PRRs). They recognize polysaccharides, LPS, peptidoglycans, bacterial DNA, double stranded viral RNA and other molecules not

normally found on the surface of multicellular organisms[38]. The PRRs can be soluble components like complement protein C3, lectins and various other humoral innate components, or they can be expressed as receptors on phagocytes and other cells of the immune system[38]. Toll receptors are type I transmembrane proteins that are evolutionarily conserved between insects and higher vertebrates. A homologous family of Toll receptors, the Toll-like receptors (TLRs) may have been the first PRRs involved in host defense[39]. Following recognition of PAMPs, they initiate intracellular signal transduction that results in the expression of genes involved in immune responses[40], such as antimicrobial peptides, antiviral interferons (IFNs) and transcription factors that result in the activation of interferon regulatory factors (IRFs)[41].

Cytokines: Activation of the pathogen-sensing receptors expressed by macrophages and dendritic cells induces the production of cytokines that activate the cellular components of the innate immune system. Cytokines are small proteins released from various cells and they induce responses by binding to specific receptors; they have important local and systemic effects, initiating and regulating different responses according to the cytokine family they belong to. Cytokines play a crucial role in the induction of effector cells to the sites of the original stimulus (infection, inflammation) and in the activation of the acute-phase response. Components of the acute phase response are then produced and have broad specificities for PAMPs, aiding in the host's immune defense[41]. Numerous cytokines have been isolated and characterized in fish, such as tumor necrosis factor-α (TNF- α), interleukins and chemokines[42].

Lysozyme: Fish lysozyme is a mucopeptide hydrolase of leucocytic origin, mainly distributed in the head kidney, gills, skin, gastrointestinal tract and eggs. Lysozyme separates peptidoglycan layers (linkages between N-acetylmuramic acid and N-

acetylglucosamine) in the cell walls of Gram-positive bacteria, preventing them from invading the host cells. Gram-negative bacteria are not directly damaged by lysozyme but it degrades chitin present in cell walls of fungi and the exoskeletons of some invertebrates[43]. Lysozyme was reported in sturgeon species such as the sterlet, diamond sturgeon, starry sturgeon and beluga sturgeon. There were species differences but in general, serum had the lowest level of lysozyme, followed by liver, spleen and kidney, which had the highest levels in all sturgeon species studied[44].

Complement: In lower and higher vertebrates, the complement system and lectins are other important components of the innate system[45]. Complement proteins circulate in an inactive form. In the presence of a pathogen, they interact with each other and give rise to complement pathways, facilitating opsonization, phagocytosis and inflammatory responses that result in pathogen killing[41]. Teleost fish complement includes the classical and alternative pathways similar to those present in higher vertebrates. In rainbow trout, both pathways have structurally and functionally similar terminal membrane attack complexes (MAC) as humans[45].

Natural antibodies: Natural antibodies are produced in the complete absence of exogenous antigenic stimulation and provide early and broad protection against pathogens[40]. In mammals, they are usually encoded by germline variable genes without extensive mutations and have a wide range of binding avidities[40]. Natural antibodies have been identified in the sera of normal, non-immunized humans, mice and in some teleosts. In general, teleost fish have IgM as their immunoglobulin responsive to antigen and are capable of eliciting effective specific humoral antibody responses against various antigens[40]. The intensity of this response has been shown to vary between different species of teleosts under different environmental conditions and relatively high

levels of natural antibodies are often seen in fish serum. Natural antibodies are thought to be a crucial link to the adaptive immune response[40].

Cellular components: Bony fish have well characterized macrophages and granulocytes. Neutrophils (acidophils) produce reactive oxygen species and macrophages have bactericidal properties due to their phagocytic ability and opsonization[46]. Despite the similar nomenclature used in human hematology when classifying teleost leucocytes according to their affinities for acid or basic dyes, a similar function may not be applicable to the granulocytes of teleosts[40]. This occurs in sturgeon, where despite the morphology of blood cells being similar to that of other fish species[47], shovelnose sturgeon (Scaphirhynchus platorynchus) neutrophils were classified as heterophils, partly due to the lack of peroxidases present in these cells. Myeloperoxidase (MPO) is used as an indication of phagocytic, chemotactic, and bactericidal functions of fish neutrophils in the degranulation process. This enzyme is absent in birds and in some fish species such as the shovelnose sturgeon and instead they possess alkaline phosphatase (AP) in their granulocytes. The lack of MPO to form complexes in order to reduce damage caused by free oxygen radicals and the inability to produce an oxidative burst may limit their ability to kill a wide variety of bacterial organisms[48].

1.5.4. Adaptive immune system

Adaptive immunity evolved around 500 million years ago and is characteristic of all vertebrates[49,50]. Possible causes for a conserved adaptive immunity are increased metabolic rates, coevolution with specialized parasites and genomic instability. The development of the brain and a closed circulation pose a high metabolic demand and subsequent increased food intake, exposing animals to a greater number of pathogens.

An increased metabolic rate would be accompanied by greater oxidative damage to DNA. Genome-wide duplication events lead to genomic instability and a higher probability of mutation occurrence. All these factors may have contributed to the selection for gene repair mechanisms and the need for a policing system like the immune system[51].

Despite being present in all vertebrates, agnathans (jawless vertebrates such as lampreys and hagfish) and gnathostomes (jawed vertebrates) have different molecular mechanisms for antigen recognition. Agnathans possess leucine-rich-repeat (LRR) genes in pieces that can be somatically assembled to encode variable lymphocyte receptors (VLRs) that are used for antigen recognition in adaptive immune responses. Three VLR genes (VLRA, VLRB and VLRC) have been identified in lamprey lymphocytes[49]. VLRA and VLRB are expressed on the surface of T-like and B-like lymphocytes, respectively, and VLRC is expressed by a third lymphocyte lineage. VLRA+ and VLRC+ lymphocyte lineages may be equivalent to the αβ and γδ T cell lineages in jawed vertebrates but further research is needed to assess their function. In agnathans, VLRA expressing lymphocytes develop in a thymus-like tissue (termed thymoid) present at the extremities of the gill filaments and VLRB expressing lymphocytes develop in hemopoietic tissues[49]. Lamprey B-like lymphocytes respond to antigens by differentiating into plasma cells that secrete VLRB as a multivalent protein. Reciprocal expressions of cytokines and chemokines and their receptors imply communication between these lymphocytes for productive immune responses[49,50]. Activated VLRB lymphocytes up-regulate their expression of IL-8 transcripts, possibly using this cytokine to attract and engage IL-8 receptor-bearing VLRA lymphocytes and other cell types to amplify the response to potential pathogens[50]. Agnathans lack recombination-activating genes (RAG1 and

RAG2) present in gnathostomes and recombination signal sequences for the VLR gene segments responsible for the V(D)J recombination that enables the generation of diverse repertoires of Ig-based antigen receptors. Instead, lampreys have two genes, CDA1 and CDA2, which possibly catalyze the assembly of VLR genes by a gene conversion-like mechanism, resulting in a potential antigen receptor repertoire of $>10^{14}$ antigen specificities characteristic of jawed vertebrates[49]. In jawed vertebrates, B and T lymphocytes use different mechanisms to recognize antigen. T cells have somatically diversified T cell receptors (TCR) for cell-to-cell interactions based on specific (major histocompatibility complex, MHC) recognition of non-self determinants. B cells use somatically diversified Ig genes, they have membrane-bound receptors and secrete Igs that deal with circulating antigen products[49,52]. During their development in the thymus and hemopoietic tissues, respectively, T and B lymphocytes use V(D)J recombination to generate diverse repertoires of their Ig-based antigen receptors that are capable of recognizing $>10^{14}$ potential antigens. The genes encoding the key recognition receptors of this adaptive immune system (Ig, TCR and MHC genes) are present in all jawed vertebrates[49].

Igs possess V (variable) regions, which vary in structure between different Ig molecules and are involved in antigen binding. The C (constant) region of the Ig molecule interacts with effector cells and molecules. There are 5 classes of Igs (IgM, IgG, IgD, IgA and IgE) in mammals but there is considerable heterogeneity in teleosts within different species. IgM has been documented[53] in fish, IgZ/IgT in zebrafish[54] and rainbow trout[55] and IgD in channel catfish (Ictalurus punctatus)[56] and sturgeon[57]. Sturgeon are an intermediate group between cartilaginous fish and teleosts and their immunoglobulin structure shares similarities with both groups. There are three major

types of organization of the Ig loci, denoted multicluster (as in the elasmobranchs), minimalistic (in birds) and translocon type (in mammals)[2]. The translocon type of organization consists of multiple alongside (tandem) duplications of gene segments within one locus, ensuring that all components remain under the control of one set of regulatory elements[58]. The Siberian sturgeon (A. baeri) has been described as having one Ig class (similar to IgM), which forms different polymers of the basic unit H_2L_2[59]. The light chain gene (IgL) has a translocon type of organization of the IgL locus with many V_L segments and with several (at least seven) J_L segments upstream of the C_L segment(s)[2,59]. The intermediate evolutionary state of sturgeon between cartilaginous fish and teleosts is supported by the similarities of the C_L sequence to the former group and of the V_L to the latter. The IgH loci of sturgeon and tetrapod species have a similar (translocon) organization differing from that of cartilaginous fish (multicluster). The Siberian sturgeon has an IgH locus with a translocon type of organization. Sturgeon C_H sequences are most closely related to those of elasmobranchs, however[59].

1.5.5. Antiviral responses in fish

In mammals, TLRs such as TLR-7 and TLR-9 from virus infected cells activate different transcription factors that are important for inducing antiviral type I interferons (IFNs)[41]. IRFs are a family of important transcription factors involved in the interferon (IFN) response and several members have now been described (interferon regulatory factor 1 to IRF-9)[60]. Gene knockout mice show that IRFs are involved in host response to viral and bacterial infections, development and activation of lymphocytes, susceptibility to oncogenic transformation, regulation of the cell cycle and apoptosis[59]. IFNs are inducible cytokines first described in mammals in 1957[61,62]. They have been

divided into three types according to their biological and structural properties: type I (IFN α, β, ω, κ, ε), type II (IFN-γ) and type III (IFN-λ, formerly IFN-like cytokine)[63]. Type I IFNs are also known as viral IFNs and are induced by virus infection. Type II IFN is known as immune IFN and is induced by mitogenic or antigenic stimuli[64]. Type III IFNs exhibit a type I IFN-like antiviral response in a restricted subset of cells[65]. Antiviral type I IFNs stimulate the production of cytokines and chemokines that recruit antigen-presenting cells (APCs) such as dendritic cells and macrophages, as well as lymphocytes and natural killer (NK) cells. After phagocytizing viral particles, APCs present viral antigenic peptides to naive $CD8^+$ T cells (cytotoxic T-lymphocytes, CTLs) and $CD4^+$ T cells (T helper cells) in association with MHC class I and class II molecules, respectively. Activated $CD8^+$ T cells (virus-specific effector CTLs) then kill the virus-infected cells, along with the NK cells[41]. Type II IFN is produced by activated T cells and NK cells. This in turn stimulates other NK cells, activates macrophages and induces specific cytotoxic immunity based on the recognition of cell surface-bound viral antigens expressed in association with MHC proteins, playing an important role in antiviral and antimicrobial immune responses[60].

Fish are able to mount strong and protective immune responses to many viruses, as evidenced by vaccine efficacy. Both IRFs and IFNs have been described in fish. IRF-1 and IRF-2 have been studied in teleosts such as the rainbow trout[66] and Atlantic cod (Gadus morhua)[67]. IRF-1, IRF-2 and IRF-5 have also been characterized in phylogenetically primitive species like the chondrostean paddlefish (Polyodon spathula)[68]. Fish IFNs have been often compared to type I mammalian IFNs, although their receptor is closer to the type III interferon receptors[63]. In teleosts, IFN production was first demonstrated in vivo in rainbow trout, following viral infection with Egtved

virus, the causal agent of viral hemorrhagic septicemia (VHS)[69]. Since then, IFN production has also been studied in several teleost species such as salmonids[70], zebrafish[71] and channel catfish[72]. An important aspect of the IFN-induced antiviral activity is that it does not imply an antibody response and, therefore, fish larvae would presumably not need to be immunocompetent to acquire protection[70].

Viral pathogens have been described in sturgeon species. In white sturgeon, iridovirus (WSIV), adenovirus (WSAV) and herpesvirus (WSHV) all seem to cause more serious systemic infections in younger fish (larvae and juveniles) than older fish, with important economic losses[73]. White sturgeon surviving WSIV infection had significant serum antibody end point titers ranging from 50 to 6400 compared to unexposed animals, measured by enzyme-linked immunosorbent assay (ELISA). Following intraperitoneal vaccination and boost with inactivated WSIV at 15°C, they had a 16-fold greater anti-WSIV titer than with primary vaccination[74]. These data suggest a strong antibody production following viral exposure, which may be protective in the case of future infection[74]. However, this response took 6-9 weeks to emerge, whereas studies with sturgeon Huso huso at 22°C revealed a significant induction of anti-Aeromonas hyrophila antibodies within 15 days[75].

Considering that in mammals, IRFs are also involved in host response to bacterial infections[60] and that sturgeon are impacted by bacterial diseases such as Flavobacterium spp and A. hydrophila[75] and A. salmonicida[76], understanding how sturgeon respond to these pathogens at different temperatures is important.

1.5.6. Skin immunity in fish

Skin mucus is an important site of immunological interaction between the fish

and its environment. It acts as a first barrier against pathogens and contains many of the innate factors listed above as well as components of adaptive immunity such as immunoglobulins. Acute inflammatory responses, the first step in an innate response, are elicited in teleosts after induction by various stimuli such as inoculation of bacteria, exposure to parasites and wounding. These responses are characterized by neutrophilia, monocytosis and accumulation of neutrophils and macrophages at the site of the stimulus. Tissue repair occurs after phagocytosis of the antigen molecules and the necrotic tissue. Along with increased risk of infection, a particularly important consequence of skin injury in fish is an immediate osmotic imbalance. Epidermal healing occurs by adjacent cell migration within hours and full dermis regeneration takes place in 3 to 4 weeks[1]. Inflammatory and regenerative responses following mechanical tissue damage and natural infection by M. viscosus were characterized in Atlantic salmon and rainbow trout[77]. Cytokines such as IL-1β, IL-8 and IL-10 as well as TLRs were useful indicators of inflammation in teleosts. These inflammatory cytokines were up-regulated following damage and infection, being significantly higher in the infected fish. Molecules coding for cellular proliferation and differentiation (tumor growth factor-β (TGF-β)), degradation of extracellular matrix (metalloprotease 2 (MMP-2)), fibrotic responses (connective tissue growth factor (CTGF)) and tissue regeneration were also induced following infection and tissue damage in these fish. To my knowledge, no skin studies have been done in sturgeon and it would be relevant to assess how skin immunity helps protect these fish from external stressors and pathogens.

1.6. Current investigation

1.6.1. Problem

Most sturgeon species originate from river and estuarine systems heavily impacted by anthropogenic stressors. Understanding the timing of immune development in larvae and juvenile sturgeon and establishing a baseline for their immune responses to external factors (such as temperature variation and parasite exposure) will provide insight into how these stressors may potentially affect sturgeon immune responses. It may also facilitate husbandry at sturgeon hatcheries and improve early stage survival and growth rates.

1.6.2. Central hypothesis

Sturgeon are capable of mounting an overall effective immune response to outside stimuli similar to that present in teleosts, despite their primitive status.

1.6.3. Specific aims and objectives

Aim 1: To characterize the morphology and cell composition of the kidney, meningeal myeloid tissue, spleen and thymus in Atlantic sturgeon (A. oxyrinchus oxyrinchus) embryos, larvae and juveniles.

Objectives: To understand when the kidney, meningeal myeloid tissue, spleen and thymus appear and how they develop in larvae and juvenile sturgeon.

Working hypothesis: It is hypothesized that sturgeon kidney, meningeal myeloid tissue, spleen and thymus appear and develop at a similar life stage or later in life when compared to teleost species such as Atlantic salmon and rainbow trout.

Rationale: The culture of larvae is considered to be one of the most critical and difficult

stages in intensive sturgeon farming, often associated with a high mortality rate during and after yolk sac absorption and onset of feeding[78]. Studying the development of relevant immune organs in sturgeon would help in the understanding of the overall immune competence in these fish during the critical stages.

Strategy: Eggs, larvae and juvenile Atlantic sturgeon would be collected at a sturgeon hatchery at different time points and processed for light and electron microscopy (LM and TEM, respectively). The kidney, meningeal myeloid tissue, spleen and thymus would be characterized morphologically for the different age groups. Ten fish per group would be analyzed (5 for LM and 5 for TEM) per time point.

Aim 2: To characterize the morphology and cell composition of sturgeon meningeal myeloid tissue, spleen and thymus, reared at different temperatures (11°C and 20°C), before and after exposure to an ectoparasitic copepod (Dichelesthium oblongum).

Objectives: To understand sturgeon immune responses to different external stressors and conditions such as temperature changes and exposure to parasites.

Working hypotheses: It is hypothesized that the fish kept at 20°C should have more developed immune organs than those kept at 11°C, the latter relying more heavily on innate immunity (with higher production of innate immune cells such as heterophils). Smaller fish should have less developed immune organs than larger fish of the same age.

Rationale: Water temperature is the single most important factor influencing development and growth in fish[79]. Size also influences functional immune responses in fish and a stronger and more developed immunological response occurs in larger fish within a group of animals of the same age[30]. Studying how different water temperatures affect immune responses of different sized sturgeon of the same age would be useful for

improving husbandry practices in hatcheries and farms by adjusting the water temperature at which their fish are kept.

Strategy: Same aged juvenile shortnose sturgeon (A. brevirostrum) would be divided into 2 groups according to size and distributed among eight 150L freshwater flow-through circular tanks, with 30 fish per tank and 12-hour light/dark circadian cycles. Four tanks would be kept at 11°C and the remaining tanks at 20°C. At 3 weeks, 6 weeks and 14 weeks after temperature change, 6 fish would be sampled from each tank (3 for LM and 3 for gene expression) and the kidney, meningeal myeloid tissue, spleen, thymus and skin collected for all fish. The kidney, meningeal myeloid tissue, spleen, thymus would then be characterized morphologically and the spleen and skin analyzed for expression of relevant immune genes involved in growth and development: interferon regulatory factors 1 and 2 (IRF-1 and IRF-2) and matrix metalloprotease 9 (MMP-9).

1.7. References

1. Iwama, G. & Nakanishi, T., 1996. The Fish Immune System: organism, pathogen, and environment. Academic press. USA. Pp. 1, 2, 69, 70, 133, 342.

2. Lundqvist, M., Bengten, E., Stromberg, S. & Pilstrom, L., 1996. Ig light chain gene in the Siberian sturgeon (Acipenser baeri): Implications for the evolution of the immune system. J. Immunol. 157, 2031-2038.

3. Birstein, V.J. 1993. Sturgeons and Paddlefishes: Threatened fishes in need of conservation. Conservation Biology. 7, 773-787.

4. Bronzi, P., Rosenthal, H., & Gessner, J. 2011. Global sturgeon aquaculture production: An overview. J. Appl. Ichthyol. 27, 169-175.

5. Van Den Avyle, M.J. 1984. Species profiles: 1ife histories and environmental requirements of coastal fishes and invertebrates (South At1antic) - At1antic sturgeon. U.S. Fish Wildl. Serv. FWS/OBS-82111.25. U.S. Army Corps of Engineers, TR EL-82-4.

6. National Marine Fisheries Service. 2007. Status Review of Atlantic Sturgeon (Acipenser oxyrinchus oxyrinchus). Atlantic Sturgeon Status Review Team, National Marine Fisheries Service, National Oceanic and Atmospheric Administration (NMFS).

7. Stein, A. Friedland, K. & Sutherland, M., 2004. Atlantic Sturgeon marine distribution and habitat use along the Northeastern Coast of the United States. Trans. Am. Fish. Soc. 133, 527-537.

8. Kynard, 1997. Life history, latitudinal patterns, and status of the shortnose sturgeon, Acipenser brevirostrum. Environ. Biol. Fishes. 48, 319-334.

9. National Marine Fisheries Service. 1998. Final recovery plan for the shortnose sturgeon (Acipenser brevirostrum). Prepared by the Shortnose Sturgeon Team for the National Marine Fisheries Service, Silver Spring, Maryland.

10. Dadswell, M.J. 1979. Biology and population characteristics of the shortnose sturgeon, Acipenser brevirostrum LeSueur 1818 (Osteichthyes: Acipenseridae), in the Saint John River estuary, New Brunswick, Canada. Can. J. Zool. 57, 2186-2210.

11. Gilbert, C.R. 1989. Species profiles: life histories and environmental requirements of coastal fishes and invertebrates (Mid-Atlantic Bight)-Atlantic and shortnose sturgeons. U.S. Fish Wildl. Serv. Biol. Rep. 82(11.122). U.S. Army Corps of Engineers TR EL82-4. 28 pp.

12. Jenkins, W.E., Smith, T.I.J., Heyward, L.D. & Knott, D.M. 1993. Tolerance of shortnose sturgeon, Acipenser brevirostrum, juveniles to different salinity and

dissolved oxygen concentrations. Proc. Annu. Conf. Southeast. Assoc. Fish Wildl. Agen. 47, 476-484.

13. Ludwig, A., 2008. Identification of Acipenseriformes species in trade. J. Appl. Ichthyol. 24 (Suppl. 1), 2-19.

14. Barton, B.A., Bollig, H., Hauskins, B.L. & Jansen, C.R. 2000. Juvenile pallid (Scaphirhynchus albus) and hybrid pallid x shovelnose (S. albus×platorynchus) sturgeons exhibit low physiological responses to acute handling and severe confinement. Comp. Biochem. Physiol. A Physiol. 126, 125-134.

15. Haukenes, A.H., Barton, B.A. & Bollig, H. 2008. Cortisol responses of pallid sturgeon and yellow perch following challenge with lipopolysaccharide. J. Fish Biol. 72, 780-784.

16. Baker, D., Wood, A., Litvak, M. & Kieffer, J., 2004. Haematology of juvenile Acipenser oxyrhynchus and Acipenser brevirostrum at rest and following forced activity. J. Fish Biol. 66, 208-221.

17. Leaman, B.M. & Beamish, R.J. 1984. Ecological and management implications of longevity in some northeast Pacific groundfishes. International North Pacific Fisheries Commission Bulletin. 42, 85-97.

18. McCormick, M.I. 1998. Behaviorally induced maternal stress in a fish influences progeny quality by a hormonal mechanism. Ecology. 79, 1873-1883.

19. Gagliano, M. & McCormick, M.I. 2007. Maternal condition influences phenotypic selection on offspring. J. Anim. Ecol. 76, 174-182.

20. Berkeley, S.A., Chapman, C. & Sogard, S.M. 2004. Maternal age as a determinant of larval growth and survival in a marine fish, Sebastes melanops. Ecology. 85, 1258-1264.

21. Swain, P. & Nayak, S.K. 2009. Review: Role of maternally derived immunity in fish. Fish Shellfish Immunol. 27, 89-99.

22. Wang, Z. & Zhang, S. 2010. The role of lysozyme and complement in the antibacterial activity of zebrafish (Danio rerio) egg cytosol. Fish Shellfish Immunol. 29, 773-777.

23. Kim, B., Nam, B., Kim, J., Park, H., Song, J. & Park, C. 2011. Molecular characterization and expression analysis of a fish-egg lectin in rock bream, and its response to bacterial or viral infection. Fish Shellfish Immunol. 31, 1201-1207.

24. Yousif, A.N., Albright, L.J. & Evelyn, T.P.T. 1994. In vitro evidence for the antibacterial role of lysozyme in salmonid eggs. Dis. Aquat. Organ. 19, 15-19.

25. Zhang, S., Sun, Y., Pang, O. & Shi, X. 2005. Short Communication: Hemagglutinating and antibacterial activities of vitellogenin. Fish Shellfish Immunol. 19, 93-95.

26. Shi, X., Zhang, S. & Pang, O. 2006. Short communication: Vitellogenin is a novel player in defense reactions. Fish Shellfish Immunol. 20, 769-772.

27. Garcia, J., Munro, F.S., Monte, M.M., Fourrier, M.C.S., Whitelaw, J., Smail, D.A. & Ellis, A.E. 2010. Atlantic salmon (Salmo salar L.) serum vitellogenin neutralizes infectivity of infectious pancreatic necrosis virus (IPNV). Fish Shellfish Immunol. 29, 293-297.

28. Swain, P. Dash, S. Bal, J., Routray, P., Sahoo, P.K. Sahoo, S.K., Saurabh, S., Gupta, S.D. & Meher, P.K. 2006. Passive transfer of maternal antibodies and their existence in eggs, larvae and fry of Indian major carp, Labeo rohita (Ham.). Fish Shellfish Immunol. 20, 519-527.

29. Olsen, Y.A. & Press, C.M. 1997. Degradation kinetics of immunoglobulin in the egg, alevin and fry of Atlantic salmon, Salmo salar L., and the localization of immunoglobulin in the egg. Fish Shellfish Immunol. 7, 81-91.

30. Johnson, K.A., Flynn, J.K. & Amend, D.F. 1982. Onset of immunity in salmonid fry vaccinated by direct immersion in Vibrio anguillarum and Yersinia ruckeri bacterins. J. Fish Dis. 5, 197-205.

31. Fänge, R. 1977. Size relations of lymphomyeloid organs in some cartilaginous fish. Acta Zool. 58, 125-128.

32. Fänge, R. & Sundell, G. 1969. Lymphomyeloid tissues, blood cells and plasma proteins in Chimaera monstrosa (Pisces, Holocephali). Acta Zool. 50, 155-168.

33. Fänge, R. 1986. Lymphoid organs in sturgeons (Acipenseridae). Vet. Immunol. Immunopathol. 12, 153-161.

34. Lange, M.A. Govyadinova, A.A. & Khrushchev, N.G. 2000. Study on localization of hemopoietic tissue in sturgeon. Rus. J. Dev. Biol. 31, 372–376.

35. Tatner, M.F. & Manning, M.J. 1983. Growth of the lymphoid organs in rainbow trout, Salmo gairdneri from one to fifteen months of age. J. Zool. 199, 503-520.

36. Lam, S.H., Chua, H.L., Gong, Z., Wen, Z., Lam, T.J., & Sin, Y.M. 2002. Morphologic transformation of the thymus in developing zebrafish. Dev. Dyn. 225, 87-94.

37. Medzhitov, R. & Janeway, C.A.Jr. 1997. Innate immunity: the virtues of a nonclonal system of recognition. Cell. 91, 295-298.

38. Magnadottir, B. 2006. Innate immunity of fish (overview). Fish Shellfish Immunol. 20, 137-151.

39. Murphy, K., Travers, P. & Walport, M., 2008. Janeway's Immunobiology. 7th edition. Garland Science. USA. Pp. 714-716.

40. Whyte, S.K. 2007. The innate immune response of finfish: A review of current knowledge. Fish Shellfish Immunol. 23, 1127-1151.

41. Murphy, K. 2012. Janeway's Immunobiology. 8th edition. Garland Science. USA. Pp. 48-49, 89, 90, 99-101, 109-113.

42. Secombes, C.J., Wang, T., Hong, S., Peddie, S., Crampe, M., Laing, K.J., Cunningham, C. & Zou, J. 2001. Cytokines and innate immunity of fish. Dev Comp Immunol. 25, 713-723.

43. Saurabh, S. & Sahoo, P.K. 2008. Lysozyme: an important defence molecule of fish innate immune system. Aquaculture Research. 39, 223-239.

44. Subbotkina, T.A. & Subbotkin, M.F. 2003. Lysozyme content in organs and blood serum in various species in the Volga river. J. Evol. Biochem. Physiol. 39, 537-546.

45. Nikoskelainen, S., Lehtinen, J, & Lilius, E.M. 2002. Bacteriolytic activity of rainbow trout (Oncorhynchus mykiss) complement. Dev. Comp. Immunol. 26, 797-804.

46. Ellis, A.E. 1999. Immunity to bacteria in fish. Fish Shellfish Immunol. 9, 291-308.

47. Knowles, S., Hrubec, T.C., Smith, S.A. & Bakal, R.S. 2006. Hematology and plasma chemistry reference intervals for cultured shortnose sturgeon (Acipenser brevirostrum). Vet. Clin. Pathol. 35, 434-440.

48. Palić, D., Beck, L., Palić, J. & Andreasen, C., 2011. Use of rapid cytochemical staining to characterize fish blood granulocytes in species of special concern and determine potential for function testing. Fish Shellfish Immunol. 30, 646-652.

49. McCurley, N., Hirano, M., Das, S. & Cooper, M.D. 2012. Immune related genes underpin the evolution of adaptive immunity in jawless vertebrates. Curr. Genomics. 13, 86-94.

50. Guo, P., Hirano, M., Herrin, B.R., Li, J., Yu, C., Sadlonova, S. & Cooper, M.D. 2009. Dual nature of the adaptive immune system in lampreys. Nature. 459, 796-802.

51. Rolff, J. 2007. Why did the acquired immune system of vertebrates evolve? Dev. Comp. Immunol. 31, 476-482.

52. Zapata, A., Diez, B., Cejalvo, T., Gutierrez-de Frias, C. & Cortes, A., 2005. Ontogeny of the immune system of fish. Fish Shellfish Immunol. 20, 126-136.

53. Warr, G.W. 1995. The immunoglobulin genes of fish. Dev. Comp. Immunol. 19(1), 1-12.

54. Danilova, N., Bussmann, J., Jekosch, K. & Steiner, L.A. 2005. The immunoglobulin heavy-chain locus in zebrafish: identification and expression of a previously unknown isotype, immunoglobulin Z. Nat. Immunol. 6, 295-302.

55. Hansen, J.D., Landis, E.D. & Phillips R.B., 2005. Discovery of a unique Ig heavy-chain isotype (IgT) in rainbow trout: implications for a distinctive B cell developmental pathway in teleost fish. Proc. Natl. Acad. Sci. USA. 102, 6919-6924.

56. Bengten, E., Clem, L.W., Miller, N.W., Warr, G.W. & Wilson, M., 2006a. Channel catfish immunoglobulins: repertoire and expression. Dev. Comp. Immunol. 30, 77-92.

57. Zhu, L., Yan, Z., Feng, M., Peng, D., Guo, Y., Hu, X., Ren, L. & Sun, Y. 2014. Identification of sturgeon IgD bridges the evolutionary gap between elasmobranchs and teleosts. Dev. Comp. Immunol. 42, 138–147.

58. Hsu, E., Pulham, N., Rumfelt L. & Flajnik, M., 2006. The plasticity of immunoglobulin gene systems in evolution. Immunol. Rev. 210, 8-26.

59. Lundqvist, M., Pilstrom, L. & Strömberg, S., 1998. Ig heavy chain of the sturgeon (Acipenser baeri): cDNA sequence and diversity. Immunogenetics. 48, 372-382.

60. Sato, M., Taniguchi, T. & Tanaka, N. 2001. Survey: the interferon system and interferon regulatory factor transcription factors - studies from gene knockout mice. Cytokine Growth Factor Rev. 12, 133-142.

61. Isaacs, A. & Lindenmann, J. 1957. Virus interference. I. The interferon. Proc. Biol. Sci. 147, 258-267.

62. Isaacs, A., Lindenmann, J. & Valentine, R.C. 1957. Virus interference. II. Some properties of Interferon. Proc. Biol. Sci. 147, 268-273.

63. Verrier, E.R., Langevin, C., Benmansour, A. & Boudinot, P. 2011. Review: early antiviral response and virus-induced genes in fish. Dev. Comp. Immunol. 35, 1204-1214.

64. Samuel, C.E., 2001. Antiviral action of interferons. Clin. Microbiol. Rev. 14, 709-778.

65. Zhang, Y & Gui, J. 2012. Review: molecular regulation of interferon antiviral response in fish. Dev. Comp. Immunol. 38, 193-202.

66. Collet, B. & Secombes, C.J. 2002. Type I-interferon signalling in fish. Fish Shellfish Immunol. 12, 389-397.

67. Feng, C.Y., Johnson, S.C., Hori, T.S., Rise, M., Hall, J.R., Gamperl, A.K., Hubert, S., Kimball, J., Bowman, S. & Rise, M.L. 2009. Identification and analysis of differentially expressed genes in immune tissues of Atlantic cod stimulated with formalin-killed, atypical Aeromonas salmonicida. Physiol Genomics. 37, 149-163.

68. Xiaoni, G., Zhuo, C., Xuzhen, W., Dengqiang, W. & Xinwen, C. 2012. Molecular cloning and characterization of interferon regulatory factor 1 (IRF-1), IRF-2 and IRF-5 in the chondrostean paddlefish Polyodon spathula and their phylogenetic importance in the Osteichthyes. Dev. Comp. Immunol. 36, 74-84.

69. De Kinkelin, P. & Dorson, M. 1973. Interferon production in rainbow trout (Salmo gairdneri) experimentally infected with Egtved Virus. J. Gen. Virol. 19, 125-127.

70. Eaton, W.D. 1990. Antiviral activity in four species of salmonids following exposure to poly inosinic:cytidylic acid. Dis. Aquat. Organ. 9, 193-198.

71. Altmann, S.M., Mellon, M.T., Distel, D.L. & Kim, C.H. 2003. Molecular and functional analysis of an interferon gene from the zebrafish, Danio rerio. J. Virol. 77, 1992-2002.

72. Long, S., Wilson, M., Bengten, E., Bryan, L., Clem, L.W., Miller, N.W. & Chinchar, V.G. 2004. Identification of a cDNA encoding channel catfish interferon. Dev. Comp. Immunol. 28, 97-111.

73. Hedrick, R.P., McDowell, T.S., Groff, J.M., Yun, S. & Wingfield, W.H. 1991. Isolation of an epitheliotropic herpesvirus from white sturgeon Acipenser transmontanus. Dis. Aquat. Organ. 11, 49-56.

74. Drennan, J., LaPatra, S., Swan, C., Ireland, S. & Cain, K., 2007. Characterization of serum and mucosal antibody responses in white sturgeon (Acipenser transmontanus Richardson) following immunization with WSIV and a protein hapten antigen. Fish Shellfish Immunol. 23, 657-669.

75. Khoshbavar-Rostami, H.A., Soltani, M. & Hassan, H.M.D. 2007. Immune responses of great sturgeon Huso huso to Aeromonas hydrophila bacterin. J. Fish Biol. 70, 1931-1938.

76. Kolman, H., Kolman, R. & Siwicki, A. 2000. Non-specific defense mechanisms of Russian sturgeon (Acipenser gueldenstaedti Brandt) reared in cages. Archives of Polish Fisheries. 8, 181-192.

77. Ingerslev, H., Lunder, T. & Nielsen, M. 2010. Inflammatory and regenerative responses in salmonids following mechanical tissue damage and natural infection. Fish Shellfish Immunol. 29, 440-450.

78. Conte, F.S., Doroshov, S.I., Lutes, P.B. & Strange, E.M. 1988. Hatchery manual for white sturgeon, Acipenser transmontanus R., with application to other North American Acipenseridae. Oakland: Div. Agric. Nat. Res., University of California. Pp. 67.

79. Piper, R.G., McElwain, I.B., Orme, L.E., McCraren, J.P., Fowler, L.G. & Leonard, J.R. 1982. Fish Hatchery Management. Department of the Interior, U.S. Fish and Wildlife Service. Washington, D. C. USA. Pp. 3-4.

Chapter 2.0 Ontogeny of the immune system in Acipenserid juveniles

2.1. Abstract

Sturgeon aquaculture has increased considerably worldwide but little is known about their immunological development and competence in early life stages. Culture of larvae is considered to be one of the most critical and difficult stages in intensive sturgeon farming, often associated with a high mortality rate during and after yolk sac absorption and onset of feeding. The objective of this study was to characterize the developmental morphology of important immune organs in Atlantic sturgeon (Acipenser oxyrinchus oxyrinchus) during these periods. The meningeal myeloid tissue, spleen and thymus were characterized using light and electron microscopy and their cell populations were counted to determine percentages in different age groups, from hatching until approximately 5 months old (2895 growing degree days, °C.day (dd)). The spleen was first visible in light microscopic sections of 541 dd larvae and the meningeal myeloid tissue and thymus in 768 dd samples (approximately 400 dd after the onset of feeding). These immune organs and cell types were similar to those previously described for other fish. A cell containing elongated electron-dense granules was described but not classified. Generally, heterophil percentages were significantly higher in older fish. Younger fish had a significantly higher percentage of undifferentiated cells in studied tissues, possibly reflecting a less mature and less competent immune system. Clearly defined ultrastructural organization of these immune organs was only observed in samples from fish older than 541 dd (33 days post hatch (dph)); effective adaptive immune competence would not be expected in any of these younger life stages, but further research is needed to assess this.

Key words: Sturgeon, immunity, development, ultrastructure.

2.2. Introduction

Excessively harvested for their meat and eggs, sold worldwide as caviar, sturgeon aquaculture has increased considerably worldwide[1]. Several factors affect the quality and survival rate of fish larvae, such as those inherent to the mother and to the environmental conditions in which they are reared. The maternal transfer of non-specific immune factors such as lysozyme and complement have been reported in teleosts like zebrafish (Danio rerio)[2]. Lectins have also been documented in rock bream (Oplegnathus fasciatus)[3]. The maternal transfer of specific humoral immune factors like IgM has also been documented in Atlantic salmon (Salmo salar)[4] and carp (Cyprinus carpio)[5] among other teleosts. Similar to teleost rearing, the culture of larvae is considered to be one of the most critical and difficult stages in intensive sturgeon farming, often associated with a high mortality rate during and after yolk sac absorption and onset of feeding[6,7]. During this period, the larvae have to rely solely on innate mechanisms and possibly maternal antibody until they develop a functional adaptive immune system, which may take several months[8]. In studies of Atlantic salmon, the appearance of humoral and cellular immunity was coincident with the onset of feeding[4] and fish larvae are usually about 20 to 30 mm in length when autologous IgM was first expressed[9].

In channel catfish (Ictalurus punctatus), distinct thymic regionalization and splenic lymphoid tissue organization were not observed before 21 days post hatch (dph), suggesting limited immune responsiveness would occur prior to that[10]. However, the lack of information regarding water temperature in this and other studies makes a

comparison to colder water species such as salmonids and sturgeon more difficult. In juvenile rainbow trout (Oncorhynchus mykiss), body weight was correlated with age but the weight of immune organs such as the thymus and spleen had a higher correlation with body weight than with age[11]. Considering that size influences immune responses in fish and that a more competent immune response is expected in larger fish of the same age[12], it is hypothesized that the larger (older) sturgeon will have more developed immune organs with lower undifferentiated cell percentages than that seen in younger fish.

The lack of light microscopic and ultrastructural studies regarding sturgeon immune organs hinders our understanding of when these fish acquire a functional immune response and how they would respond at different ages to potential immune enhancement strategies. The aim of this light microscopy (LM) and transmission electron microscopy (TEM) study was therefore to determine when Atlantic sturgeon meningeal myeloid tissue, spleen and thymus first appear and to characterize their morphology and cell populations from hatching until 5 months of age.

2.3. Materials and methods

All experimental protocols followed the guidelines given in 2005 by the Canadian Council on Animal Care (http://www.ccac.ca/Documents/Standards/Guidelines/Fish.pdf) and were approved by the UPEI Animal Care Committee.

2.3.1. Fish husbandry

Atlantic sturgeon (A. oxyrinchus oxyrinchus) fertilized eggs and larvae were maintained at the Acadian Sturgeon and Caviar Inc. hatchery in Carter's Point, New Brunswick, Canada in freshwater tanks, at 11°C. Two to three hundred live 1-day post fertilization (dpf) eggs and 100 larvae at 4 dph larvae were transported to the Atlantic Veterinary College (AVC), UPEI and kept at the Aquatic Animal Facility (AAF). The eggs were maintained in a separate incubator and the larvae were maintained in a 30L freshwater fiberglass tank with a flow-through system, at 11°C and 12 hour light/dark circadian cycles. Larvae were fed ad libidum brine shrimp (Artemia franciscana) feeds per day (Appendix A – Protocol 1) from 14 dph onwards.

2.3.2. Sample collection

The sturgeon were sampled according to the schedule described in Table 2.1. For each time point, 10 animals were sampled (5 for LM and 5 for TEM, respectively). The larvae and fish were euthanized with 0.2g/l of tricaine methanesulfonate (TMS). Sample collection was based on growing degree-days, °C.day[13] (dd) and different stages of development, and it was carried out in fish maintained in AAF until 33 dph as well as at the hatcheryuntil 175 dph. At the hatchery, fish were kept at 11°C and at 22°C, allowing for 2 sample collections at the same dph but with different dd ages (Table 2.1) on dph 48 and 74.

LM: The eggs, larvae and fish were opportunistically sampled and placed in 10 % neutral buffered formalin (NBF) until further processing.

Table 2.1. A. oxyrinchus oxyrinchus sampling time points: age in hours (hpf), days post fertilization (dpf), days post hatch (dph) and growing degree days (dd).

Location of sample collection	**Age of Atlantic sturgeon**		
	hpf; dpf; dph		**dd**
Hatchery	hpf	6	-
	dpf	1	-
		2	-
		10	-
Atlantic Veterinary College	dph	2	22
		4	44
		8	88
		12	132
		14	154
		15	165
		22	242
		27	297
		29	319
		30	363
		33	541
Hatchery		48	768; 950
		74	1088; 1369
		154	2895
		175	2013
		Adult	Unknown

TEM: All eggs, larvae and fish were sampled and placed in Karnovsky's fixative (1% glutaraldehyde and 4% paraformaldehyde in 0.1M sodium phosphate buffer, pH of 7.2-7.4). The larvae collected until 768 dd were placed intact in the fixative; the spleen and a section of the head of all fish collected after 768 dd were placed in Karnovsky's fixative separately. The head section sampled was caudal to the eyes and cranial to the gill arches, including the thymus and the meningeal myeloid tissue. Based on availability, spleen and meningeal tissue samples of 3 adult sturgeon collected post spawn were processed for TEM only and used as a reference of more developed organ morphology. No thymus samples were available for collection from these adult fish.

2.3.3. Sample processing

LM: Formalin-fixed samples were trimmed, dehydrated in a graded series of ethanols to xylene, embedded in paraffin, cut in 5µm thick sections and stained with hematoxylin and eosin (H&E)[14] (Appendix A - Protocol 2). Serial sagittal sections were made for the 165 dd larvae and used as an anatomical reference of organ location. All subsequent sections were targeted based on this reference.

TEM: Tissue samples collected were initially fixed in Karnovsky's fixative for 24 hours at 4°C and then further fixed in 2% glutaraldehyde in 0.1 M sodium phosphate buffer for 1 to 2 hours at room temperature (RT) or overnight at 4°C. Sodium phosphate buffer (0.1 M) was used to wash the samples, which were then post-fixed in 1% osmium tetroxide (OsO_4) in sodium phosphate buffer for 1 to 2 hours at RT. The samples were then dehydrated in increasing ethanol concentrations (50%, 70%, 95% and absolute ethanol). The dehydrated samples were transferred to propylene oxide (PO) and

infiltrated in Epon:PO resin mixtures, then embedded in pure Epon and polymerized at 60°C (Appendix A - Protocols 3 and 4).

Semi-thin Epon sections (0.5µm thick) were cut from tissue blocks using an ultramicrotome (Reichert-Jung Ultracut E, Vienna, Austria) and glass knives (glass knifemaker Leica EMKMR2, Leica Microsystems, Ontario, Canada). Semi-thin sections were stained with 1% toluidine blue in 1% aqueous sodium borate solution, examined at the light microscopic level and the area of interest selected for cutting ultrathin sections (80 nm thick). Ultrathin sections were collected on copper grids, stained with uranyl acetate and lead Sato (Appendix A - Protocol 5) and examined using a transmission electron microscope (Hitachi TEM 7500, Nissei-Sangyo, Rexdale, Ontario), operated at 80 kV. The images observed were recorded using a AMT XR40 side mount digital camera (Advanced Microscopy Techniques, Danvers, Ma, USA).

2.3.4. Histological and ultrastructural analyses

2.3.4.1. Histological analysis

All H&E and semi-thin toluidine blue stained sections were examined for the presence of the immune organs of interest using a light microscope.

2.3.4.2. Ultrastructural analysis

When tissues of interest were present, thin sections were cut from each sample and examined using the electron microscope. The different tissues were examined at a low magnification (4000x), at a medium magnification (12000-15000x) and at a high magnification (over 20000x) as needed. For consistency and to maximize the number of cells visible in a given electron microscope field and between different organs, all cell

counts were done at the low magnification and only cells with a visible nucleus on the cut section were considered.

2.3.4.2.1. Fish size

Considering size influences fish growth and development[12], total length (cm) was measured for each fish and fish size compared between the different age groups.

2.3.4.2.2. Meningeal myeloid tissue

For each section, a differential cell count was performed and percentages of cell types relative to the total cell count were calculated. The following categories were considered, based on previous studies of sturgeon meningeal myeloid tissue[15,16]: erythrocytes/thrombocytes, heterophils, lymphocytes, eosinophils, reticular cells, undifferentiated cells and unknown or other cells. Macrophages and monocytes were not considered as a separate category due to the difficulty in accurately identifying them on TEM in the different immune organs across all the age groups. Considering that meningeal myeloid cells are scattered throughout the space between the brain and the skull[16,17], all meningeal myeloid cells present in each thin section were counted for each fish, ranging from 2 to 106 total cells per fish depending on the samples analyzed.

2.3.4.2.3. Spleen

All spleen cells were counted in the thin sections of the smaller fish. For the larger fish where the spleen samples took up numerous fields of view, 4 low magnification (4000x) fields were counted. For each section, a differential cell count was performed and percentages of cell types relative to the total cell count were

calculated. The following categories were considered, based on previous studies of sturgeon spleen[15,16]: erythrocytes/thrombocytes, heterophils, lymphocytes, eosinophils, reticular cells, undifferentiated cells and unknown or other cells, ranging from 11 to 139 total cells per fish depending on the samples analyzed.

2.3.4.2.4. Thymus

For all age groups, 4 low magnification (4000x) fields were counted per thin section for each fish. For each section, a differential cell count was performed and percentages of cell types relative to the total cell count were calculated. The following categories were considered, based on previous studies of sturgeon thymus[15,16]: erythrocytes/thrombocytes, lymphocytes, eosinophils, reticular cells, undifferentiated cells, mitotic cells, apoptotic cells and unknown or other cells, ranging from 72 to 226 total cells per fish depending on the samples analyzed.

2.3.4.2.5. Cell types

Due to the lack of ultrastructural studies of sturgeon immune organs, it was necessary to rely on past morphological and ultrastructural characterization of peripheral blood cells in order to adequately describe cell populations in hemopoietic organs in the current study (Figures 2.1-2.3). Peripheral blood cells have been described in the Chinese sturgeon (A. sinensis)[18] and shortnose sturgeon (A. brevirostrum) and their morphology was similar to that of other fish species[19].

Erythrocytes/thrombocytes: Erythrocytes were oval in shape, with a centrally positioned oval nucleus (Figure 2.1A) in a moderately electron dense, homogeneous cytoplasm generally devoid of organelles. Different maturation stages were present and

organelles such as mitochondria and ribosomes were visible in the less developed cell stages. Thrombocytes had a spindle/fusiform or oval shape, an elongated nucleus and an occasionally vacuolated cytoplasm.

Heterophils: These cells had a nucleus with varying degrees of segmentation, which increased with maturity, and numerous electron-dense granules of diverse shape, size and density throughout the cytoplasm (Figure 2.1B).

Lymphocytes: These cells had a round to oval shape, a round nucleus with increasing chromatin condensation (heterochromatin density) in the peripheral nuclear regions in the more mature stages and a high nucleus to cytoplasm ratio (Figure 2.1C).

Eosinophils: These cells had cytoplasm packed with characteristic round to oval homogeneous electron-dense granules and an eccentric nucleus in the more mature cells (Figure 2.1D).

Mesenchymal reticular cells: These cells were present in the meningeal myeloid tissue and the spleen and had a stellate appearance with branching processes (Figure 2.2A).

Epithelial reticular cells: These cells were identified by their long branching processes, elongated nucleus and presence of desmosomes at the cell surfaces connecting different reticular cells (Figures 2.1C and 2.2B).

Undifferentiated cells: These cells had a nucleus with a characteristic central region of chromatin, cytoplasmic organelles and no features particular of any given cell type (Figure 2.2C).

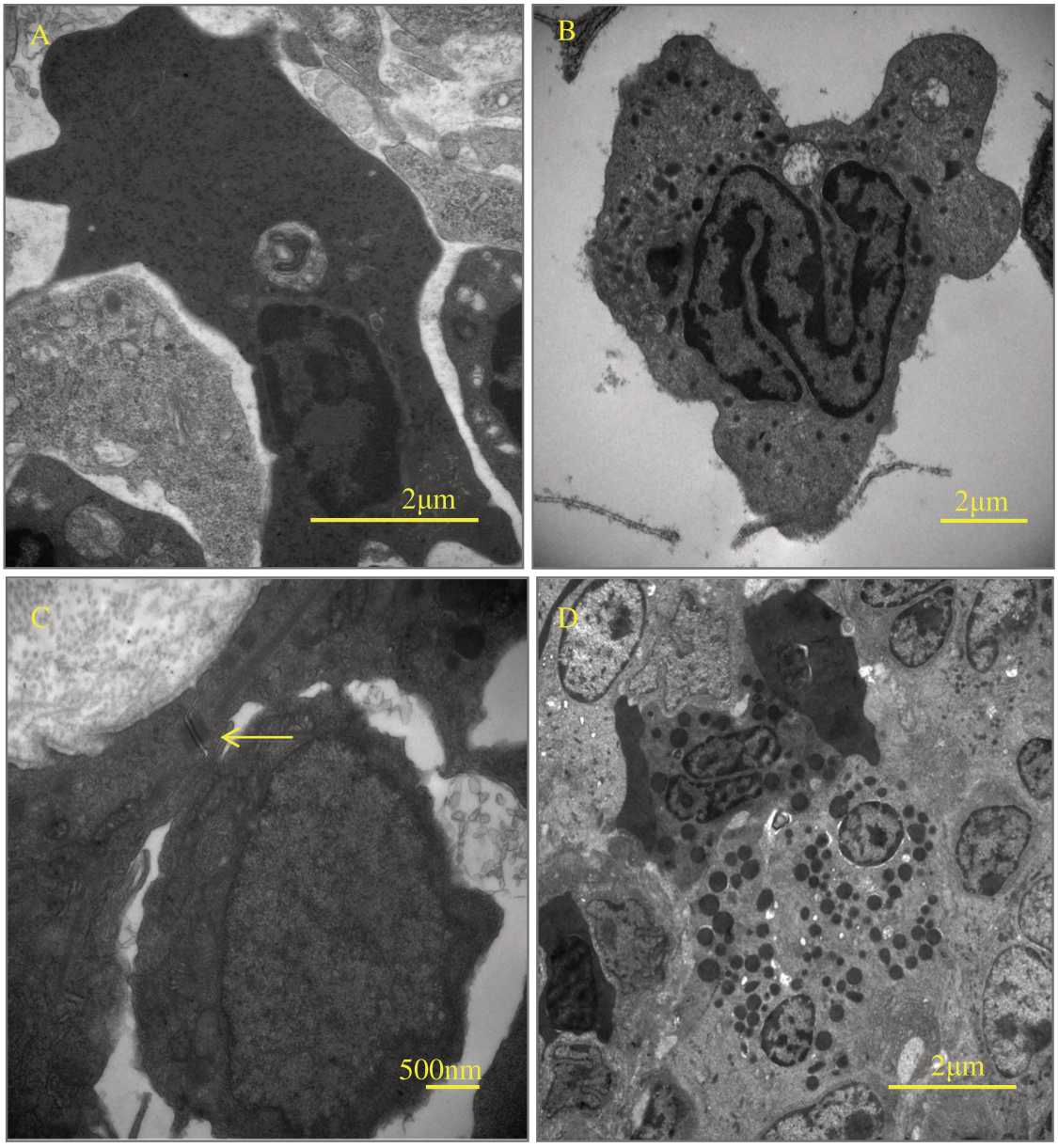

Figure 2.1. Transmission electron micrographs of A. oxyrinchus oxyrinchus hemopoietic organ cells, A) erythrocyte, B) heterophil, C) lymphocyte and a desmosome (arrow) connecting adjacent epithelial reticular cells and D) eosinophils.

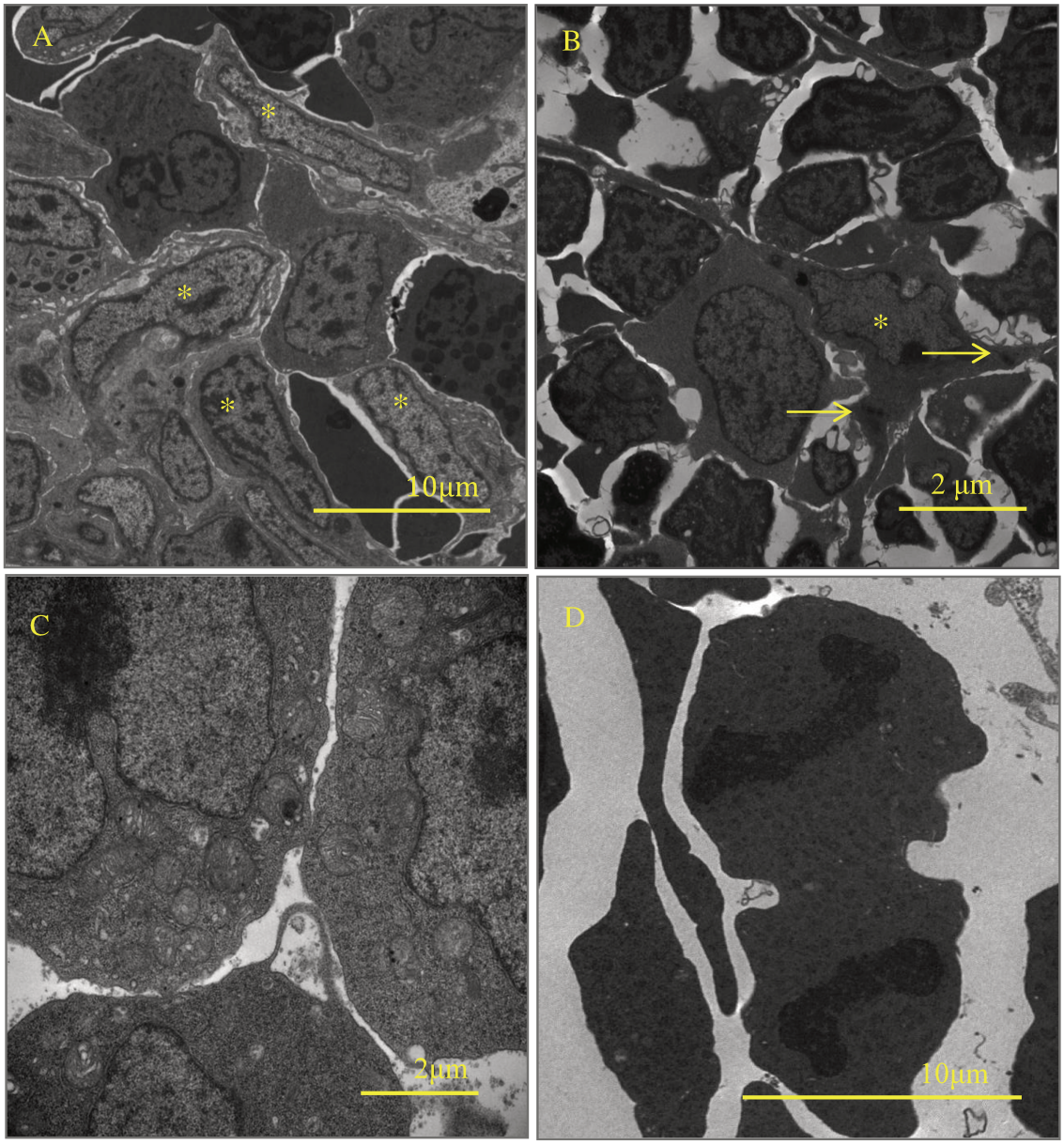

Figure 2.2. Transmission electron micrographs of A. oxyrinchus oxyrinchus hemopoietic organ cells. A) mesenchymal epithelial cells (*), B) epithelial reticular cells (*) connected by desmosomes (arrows), C) undifferentiated cells and D) mitotic cells.

Mitotic cells: These cells had a characteristic condensed chromatin pattern and were seen in different stages of cell division (Figure 2.2D).

Necrotic cells: These cells showed both nuclear and cytoplasmic swelling with cell membrane and nuclear membrane dissolution or lysis (Figure 2.3A).

Apoptotic cells: These cells show membrane blebbing, nuclear shrinkage and chromatin condensation, a reduction in cell volume and the formation of apoptotic bodies[32] (Figure 2.3B).

Other cells: Other cells included pigmented cells (Figure 2.3C) as well as granular cell types or cell stages that did not fit those previously described. One of the unclassified granular cell types was only found near the thymus of one fish in the connective tissue between the cartilaginous skull and the thymus (Figure 2.3C). Its granules were electron lucent and had a crystalline appearance (Figure 2.3D). The other unclassified granular cells were found in very low percentages in all immune organs studied (Figure 2.3E). The variably sized granules were elongated and had a homogeneous electron-dense content (Figure 2.3F).

2.3.5. Statistical analysis

Minitab 16® was used for all statistical analyses. General Linear Models were used to compare the different cell types between the age groups. One-way ANOVAs were done for the normally distributed variables (Tukey's test); for the non-normally distributed variables the Kruskal Wallis test (KW) was performed. The cut-off used for statistical significance was a p-value<0.05 (Appendix B - Table B.1). Unless specified, all values and analysis for the normally distributed variables are reported as means ± standard errors of the mean (SEM) and for the non-normally distributed variables the

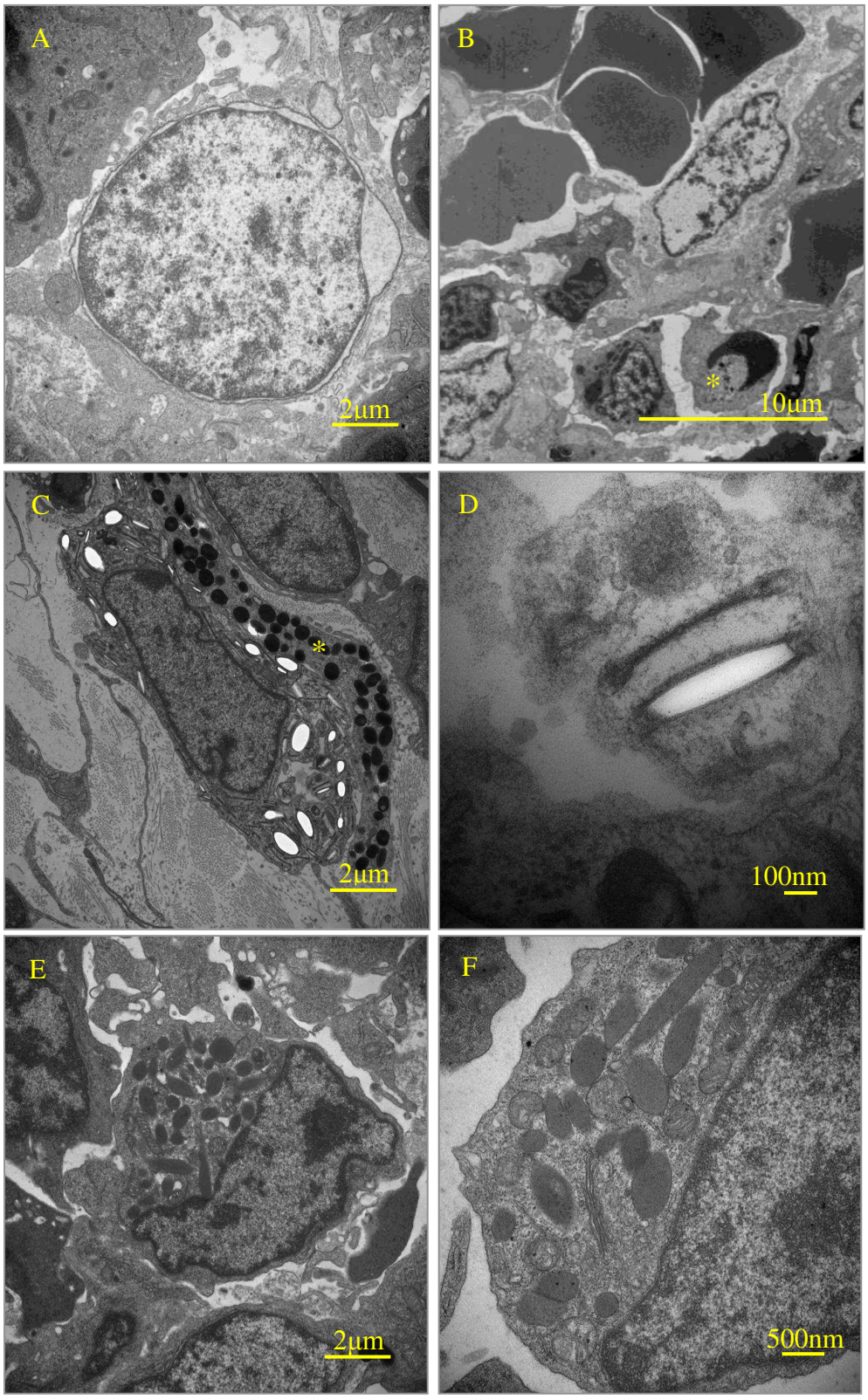

Figure 2.3. Transmission electron micrographs of A. oxyrinchus oxyrinchus hemopoietic organ cells. A) necrotic cell, B) apoptotic cell (*), C) unknown granular cell and pigmented cell (*), D) high magnification of granules in C), E) unknown granular cell and F) high magnification of granules in E).

statistical analysis was based on their medians.

2.4. Results

The sturgeon eggs kept at the AVC in the AAF did not hatch and were not processed further. In the larvae, the yolk sac was pronounced in the earlier life stages and disappeared during the transition period to exogenous feeding (at 154 dd). The serial sagittal sections of the 165 dd larvae processed for light microscopy (Figure 2.4) revealed the presence of the stomach containing food, liver, pancreas, kidney, anterior and posterior intestine (spiral valve). However, at this stage of development neither the meningeal myeloid tissue, the spleen nor the thymus were visible on any of the LM serial sections.

Based on the initial LM findings, the samples processed for TEM and analyzed were from 541 dd larvae onward: 768, 950, 1088, 1369 and 2895 dd. The 2013 dd samples were not analyzed due to the already extensive number of samples. There was a significant difference between the total length of fish in the different age groups (p-value<0.005) (Figure 2.5). The mean total length generally increased with age, except for the 950 dd group, which had the second highest mean total length (Figure 2.5).

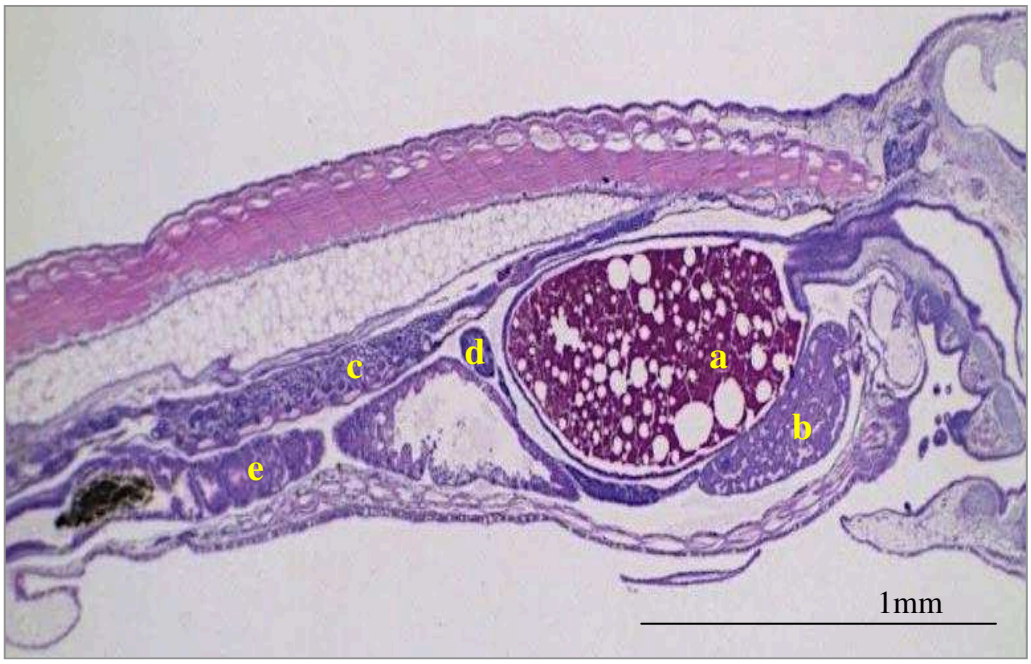

Figure 2.4. Light microscopic sagittal section of a 165 growing degree-day (dd) larva of A. oxyrinchus oxyrinchus Hematoxylin & Eosin stain. Stomach (a), liver (b), kidney (c), pancreas (d) and spiral valve (e).

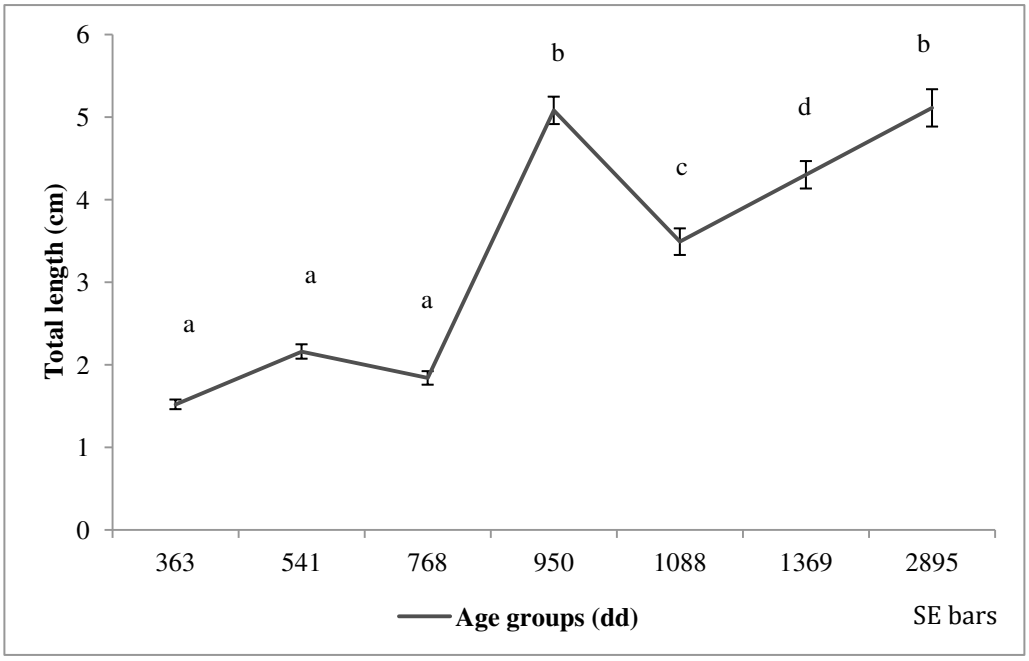

Figure 2.5. Total length in centimeters (means +/- standard error of means) of different age A. oxyrinchus oxyrinchus groups. n=5, 10; letters signify statistically significant differences between groups. dd: growing degree days.

2.4.1. Meningeal myeloid tissue

LM: The meningeal myeloid tissue was first visible in H&E sections of 768 dd larvae, located within the cranial cavity and surrounded dorso-laterally by the cartilaginous skull and medio-ventrally by the brain or spinal cord (Figure 2.6).

TEM: Unlike the findings in the H&E samples, there were no immune cells in the meningeal myeloid tissue of 768 dd old sturgeon. The space between the cartilaginous skull and the brain where the meningeal myeloid tissue is located was delineated by sporadic reticular cells (Figure 2.7A). The medio-ventral side adjacent to the brain or spinal cord was composed of small blood vessels and connective tissue, which was thickest in the oldest fish (2895 dd) (Figure 2.7B).

All p-values are indicated in Appendix table B.1. In 768 dd fish, the meningeal myeloid tissue consisted mainly of reticular cells and undifferentiated cells and most cell categories were present in 950 dd and older fish. Erythrocytes/thrombocytes were absent in the oldest group (Table 2.2):

Erythrocytes/thrombocytes (%): There was a significant difference in the erythrocyte/thrombocyte percentage between the age groups (p-value=0.009), with these cell percentages generally decreasing over time (Table 2.2).

Heterophils (%): Overall, heterophils were the most predominant cell type in the meningeal myeloid tissue, with a mean percentage for all age groups of 41.0%. There were no heterophils present in 768 dd fish, unlike the remaining age groups. There was a significant difference between the age groups (p-value=0.001). With the exception of the 1088 dd, there was a trend of larger fish having a greater heterophil percentage when compared to the smaller fish (Figure 2.8).

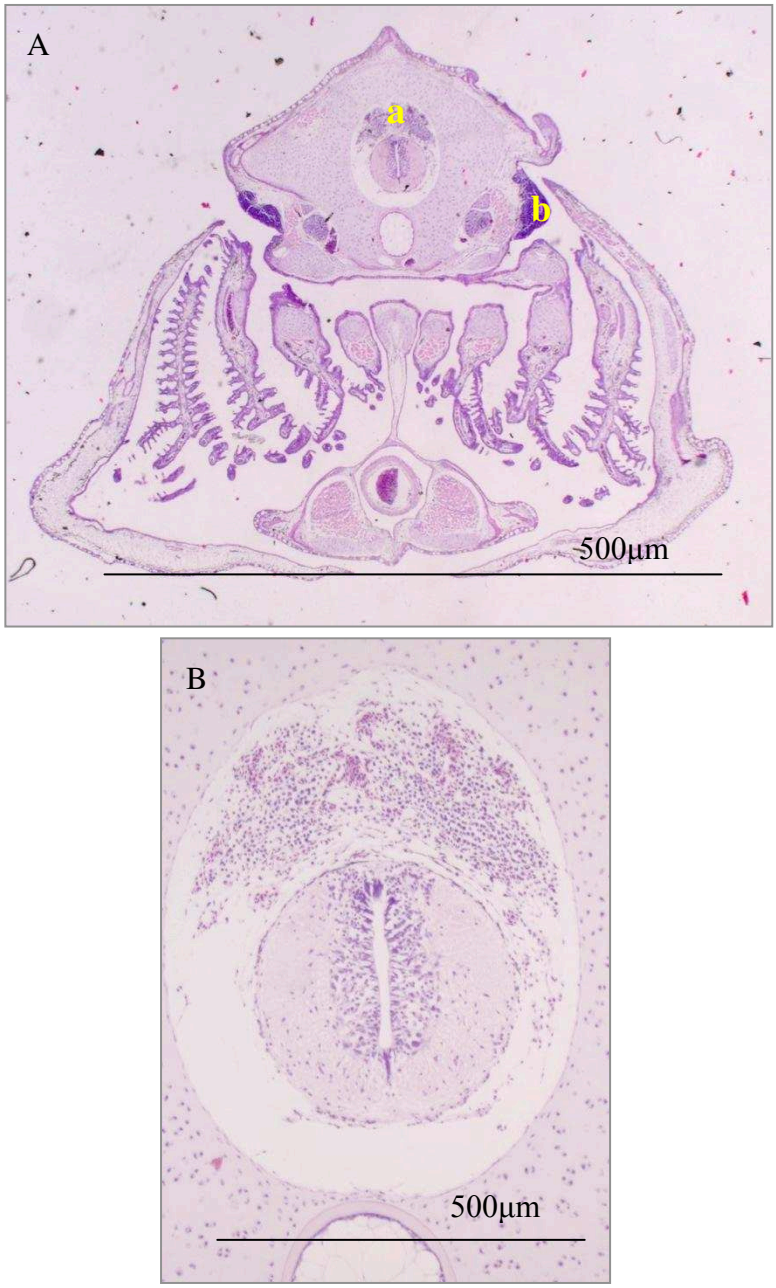

Figure 2.6. Light microscopic sections of A. oxyrinchus oxyrinchus, 768 growing degree-day (dd) larvae. Hematoxylin & Eosin stain. A) transverse section of the head: meningeal myeloid tissue (a) and thymus (b), B) higher magnification of meningeal myeloid tissue in A).

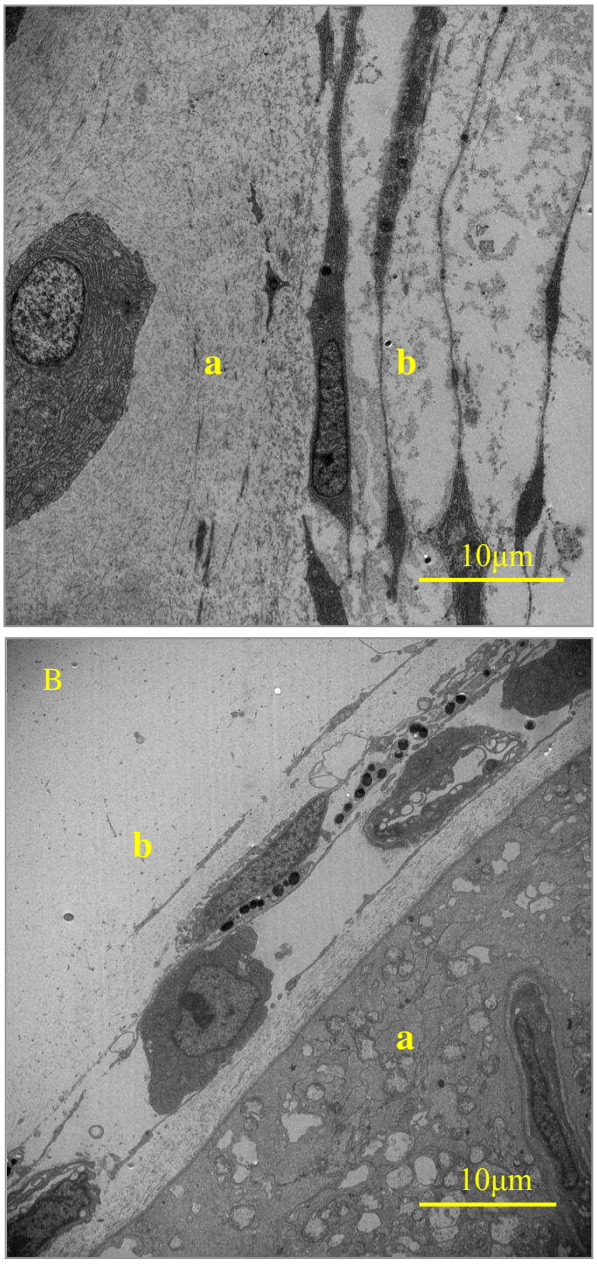

Figure 2.7. Transmission electron micrographs of A. oxyrinchus oxyrinchus, 768 dd larvae. A) skull (a) and meningeal myeloid tissue (b), B) brain (a) and meningeal myeloid tissue (b).

Eosinophils (%): There was a significant difference between the age groups (p-value=0.018), with the larger fish (950 and 2895 dd samples) having the highest mean percentages of these cells (Figure 2.8).

Lymphocytes (%): These were the least dominant immune cell type observed overall, with a mean percentage of 3.15 % across all age groups. They were absent in the 768 dd fish. There was no significant difference in lymphocyte percentages between the age groups (p-value=0.120) (Table 2.2).

Mesenchymal reticular cells (%): There was a significant difference in the reticular cell percentage between the age groups (p-value=0.007), with the youngest group having a significantly higher reticular cell percentage than the older groups (Table 2.2).

Undifferentiated cells (%): There was no significant difference between the groups (p-value=0.313 (Table 2.2).

Table 2.2. Meningeal myeloid tissue cell percentages of different A. oxyrinchus oxyrinchus age groups. n=5; dd: growing degree days; mean values ± standard errors of the mean (SEM).

Variables		768 dd	950 dd	1088 dd	1369 dd	2895 dd
Meningeal Myeloid Tissue	**Erythrocytes/ thrombocytes (%)**	0	17.3±8.2	14.2±1.4	9.8±8.4	0
	Heterophils (%)	0	43.5±12.8	23.9±10.0	47.5±10.4	65.8±8.7
	Lymphocytes (%)	0	3.7±1.1	3.9±1.3	3.3±1.2	2.9±1.1
	Eosinophils (%)	0	14.6±5.3	5.3±1.7	5.6±2.5	9.4±4.5
	Reticular cells (%)	70±30.1	11.6±4.5	9.9±4.2	7.1±2.2	4.8±1.5
	Undifferentiated cells (%)	30±30.1	8.6±1.9	31.4±10.0	24.9±8.0	16.6±4.2
	Other cells (%)	0	0.3±0.3	3.9±3.9	0.6±0.5	0.2±0.2

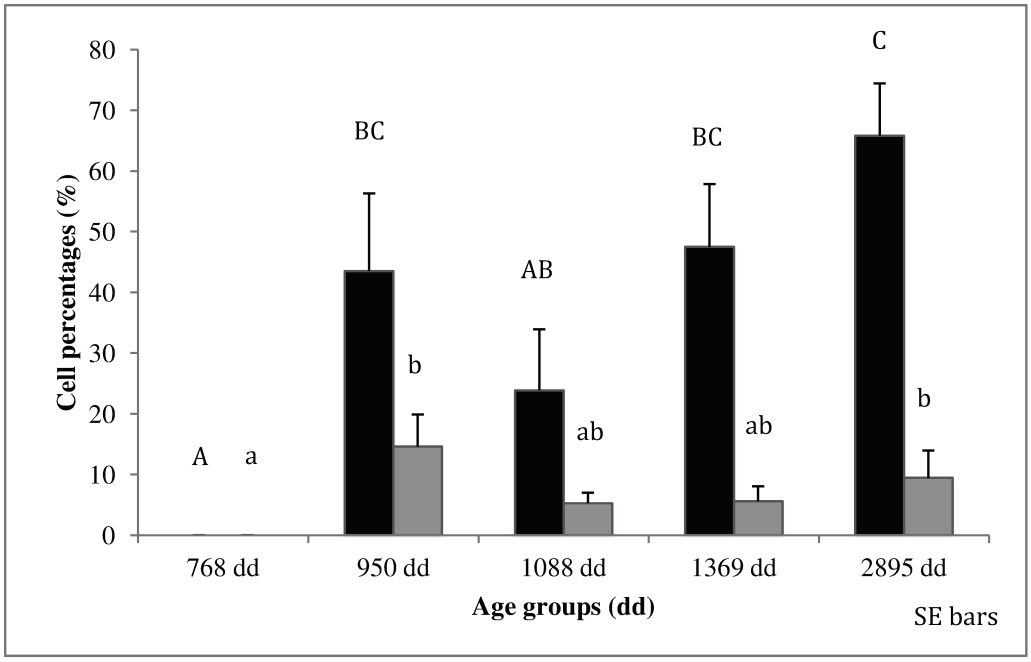

Figure 2.8. Meningeal myeloid tissue heterophil % (black bars) and eosinophil % (grey bars) of different A. oxyrinchus oxyrinchus age groups. n=5; letters signify statistically significant differences between groups. dd: growing degree days. SE: standard error bars.

2.4.2. Spleen

LM: The spleen was first visible by LM in one 33 dph (541 dd) larva (Figure 2.9A) and was consistently observed in 768 dd and older fish. The spleen had an elongated triangular shape and it was located on the ventro-lateral abdominal wall caudal to the pancreas and adjacent and lateral to the intestinal wall and caudal portion of the stomach and pancreas (Figure 2.9B). In all samples, the spleen was characterized by the presence of myeloid follicles with mainly basophilic cells (white pulp) surrounding large arteries. The erythrocytes forming the red pulp were found within ellipsoidal blood vessels and scattered between follicles. No melanomacrophage centers were visible in any of the spleen samples (Figure 2.9C).

Capsule

The splenic capsule was visible in the 768 dd samples and was composed of a thin layer of connective tissue covered by a mesothelium of simple squamous epithelium (Figure 2.10A). The epithelial cells on the peritoneal side were rounder than the cells adjacent to the intestinal wall. The connective tissue layer thickened with age but the mesothelial epithelium remained a simple squamous layer (Figure 2.10B).

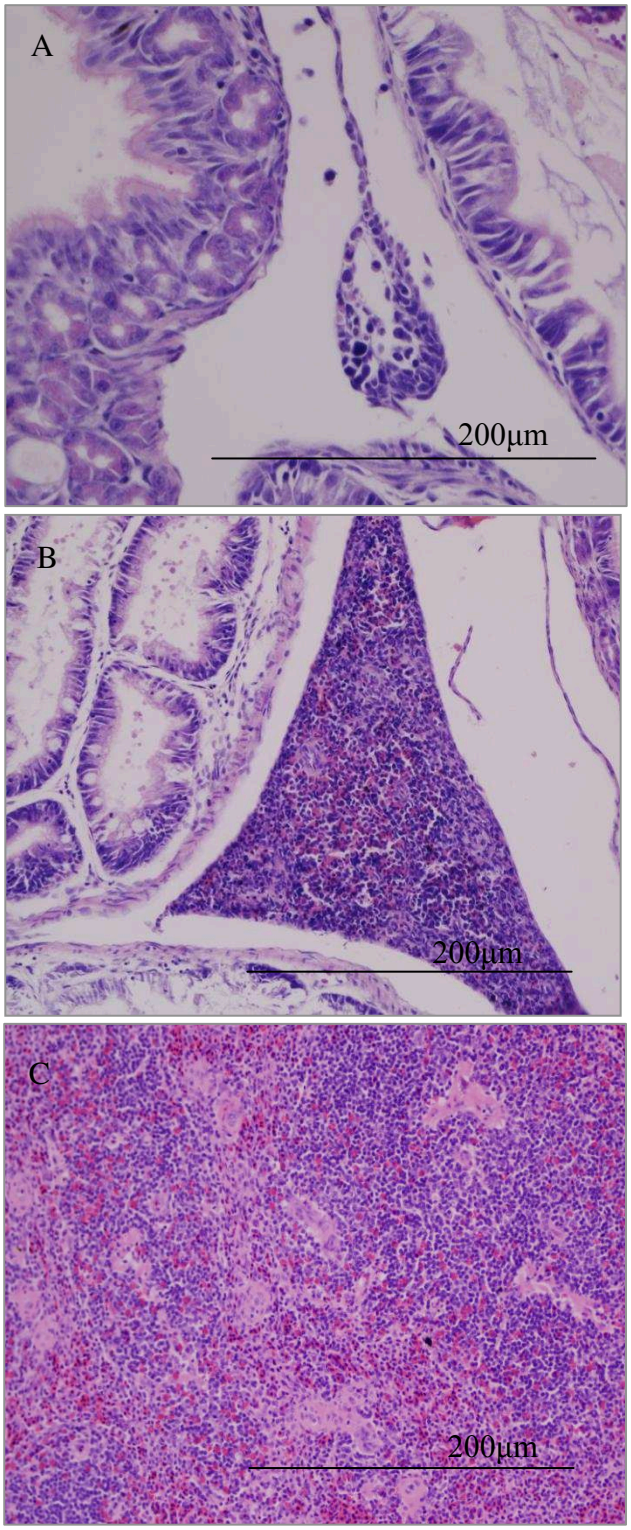

Figure 2.9. Light microscopic sagittal sections of A. oxyrinchus oxyrinchus, spleen, Hematoxylin & Eosin stain. A) 541 growing degree-day (dd) larvae, B) 950 dd and C) adult sturgeon spleen.

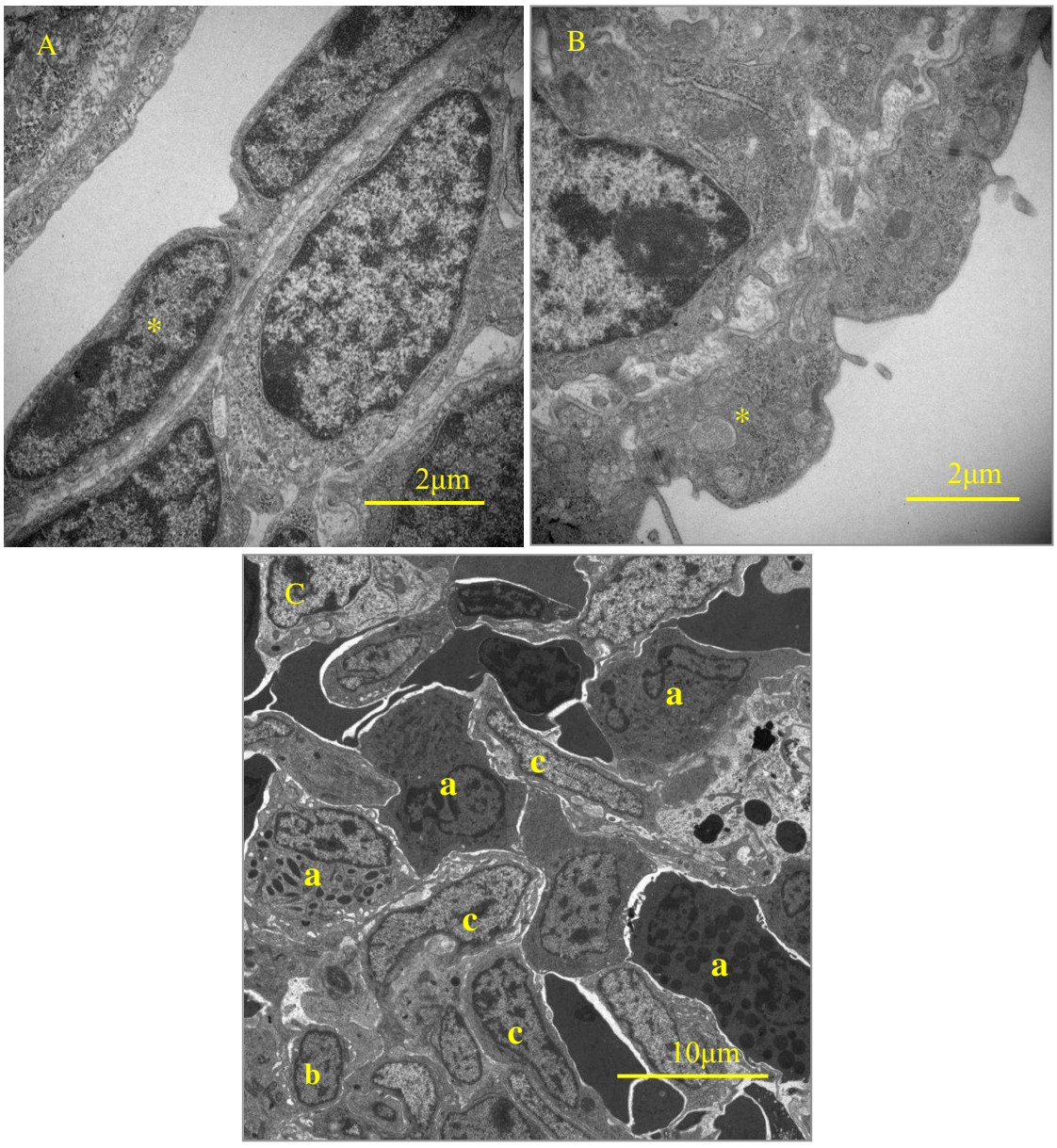

Figure 2.10. Transmission electron micrographs of A. oxyrinchus oxyrinchus spleen. A) squamous epithelium adjacent to the gastrointestinal wall of 768 growing degree-day (dd) larva (*), B) splenic squamous epithelium on the peritoneal side of 768 dd larva (*) and C) hemopoietic cells of 950 dd larva spleen (granulocytes (a), lymphocytes (b)) and mesenchymal reticular cells (c).

No predominant cell type was found in the spleen as this fluctuated over time; all cell categories were present in all age groups analyzed (Figure 2.10C) except for lymphocytes, which were absent in the 768 dd samples. All p-values are indicated in Appendix table B.1.

Erythrocytes/thrombocytes (%): There were no significant differences between the different age groups for this category (p-value=0.242) (Table 2.3).

Heterophils (%): There was a significant difference in mature heterophil percentage between the groups (p-value=0.019), generally increasing with age (Figure 2.11).

Eosinophils (%): There was no significant difference in eosinophil percentages between the age groups (p-value=0.235) (Table 2.3).

Lymphocytes (%): These cells were not observed in the spleen until 950 dd. There was a significant difference between one or more of the age groups (p-value=0.018) (Table 2.3).

Mesenchymal reticular cells (%): There was no significant difference in the splenic reticular cells between the different age groups (p-value=0.076) (Table 2.3).

Undifferentiated cells (%): There was a significant difference between the groups (p-value=0.001), with their percentage decreasing significantly with age and size (Figure 2.11).

Table 2.3. Splenic cell percentages of different A. oxyrinchus oxyrinchus age groups. n=5; dd: growing degree days; mean values ± standard errors of the mean (SEM).

Variables		768 dd	950 dd	1088 dd	1369 dd	2895 dd
Spleen	Erythrocytes/ thrombocytes (%)	20.2±8.9	23.7±1.5	36.0±6.4	20.5±6.5	40.6±0.2
	Heterophils (%)	5.4±2.4	6.6±2.7	12.2±3.0	8.2±1.3	22.3±5.2
	Lymphocytes (%)	0	2.7±1.5	3.5±2.5	12.3±5.5	5±0.8
	Eosinophils (%)	1.8±1.2	5.5±2.2	5.9±2.1	6.9±2.0	9.1±5.1
	Reticular cells (%)	6.9±6.2	13.8±8.3	5.1±2.2	28.4±5.2	7.9±3.7
	Undifferentiated cells (%)	65.0±11.4	44.2±14.0	34.3±5.2	14.2±6.5	8.2±4.0
	Other cells (%)	0.8±0.8	3.6±2.4	3.2±0.9	9.5±4.0	6.9±3.7

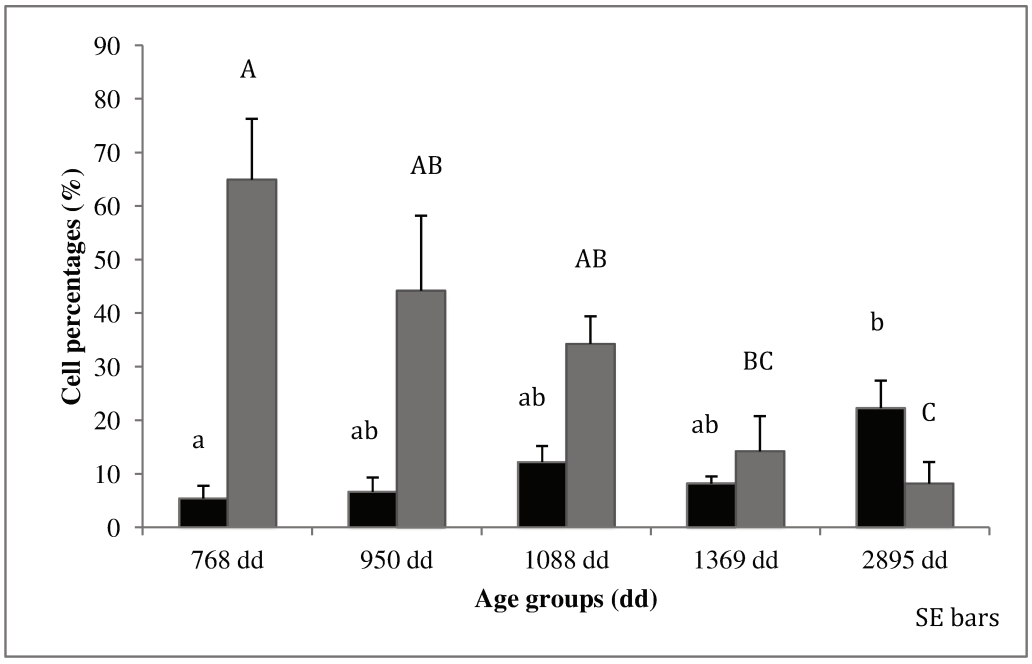

Figure 2.11. Splenic heterophil % (black bars) and undifferentiated cell % (grey bars) of different A. oxyrinchus oxyrinchus age groups. n=5; letters signify statistically significant differences between groups. dd: growing degree days. SE: standard error bars.

2.4.3. Thymus

LM: The thymus was first visible by LM in three of five 768 dd larvae (Figure 2.12A) and consistently present in 950 dd larvae and older fish (Figure 2.12B). The thymus was bilateral in all fish and consisted of an outer cortex and a less densely packed inner medulla. The thymic cortex and medulla were visible in most samples and they were filled with round, basophilic cells identified as thymocytes.

TEM: Despite the fact that the thymus was observed in three of five 768 dd fish H&E sections, none of the samples processed for TEM had a visible thymus. For this reason, this age group was not included in the statistical analysis of the thymus. In the other age groups, the thymus was separated from the water environment in the gill chamber by one to two layers of squamous epithelial cells, connected by tight junctions and desmosomes (Figure 2.13A). The cartilaginous skull and the single layer of epithelial cells adjacent to it were separated by a basement membrane (Figure 2.13B). The epithelial-reticular cells present in the thymic parenchyma created a three-dimensional mesh where the immune cells were supported (Figures 2.13C and 2.13D).

All p-values are indicated in Appendix table B.1. There were no erythrocytes or thrombocytes present in any of the age groups and there were very low percentages of heterophils and eosinophils in this tissue. The remaining cell types changed as follows (Table 2.4):

Heterophils (%): There was no significant difference between the heterophil percentages in the different age groups (p-value=0.91).

Eosinophils (%): There was no significant difference between the eosinophil percentages in the different age groups (p-value=0.88).

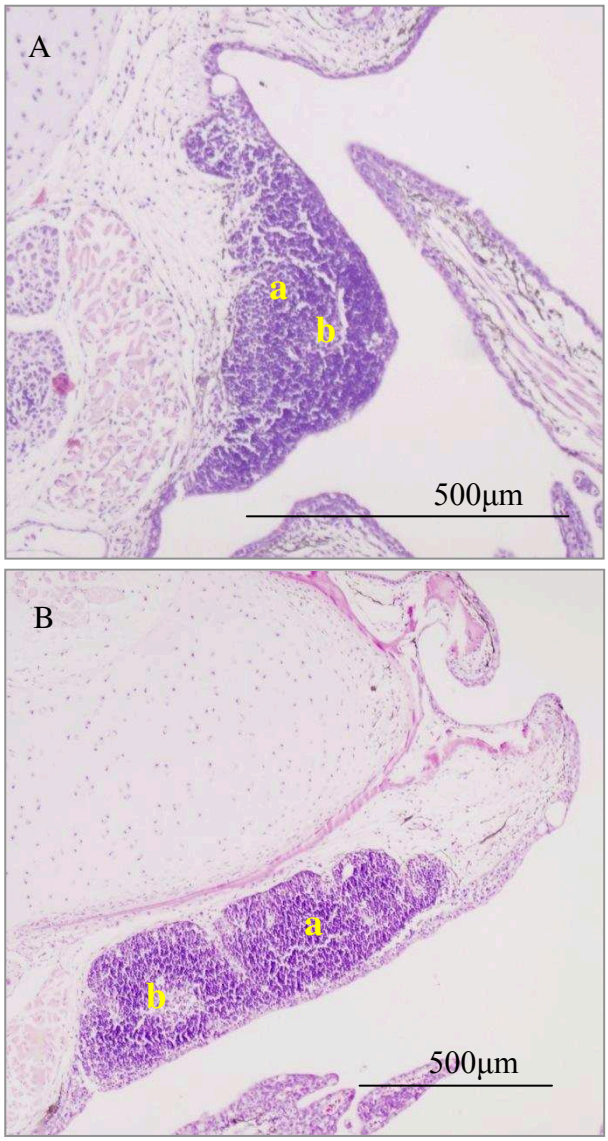

Figure 2.12. A. oxyrinchus oxyrinchus thymus, Hematoxylin & Eosin. A) 768 growing degree-day (dd) larva and B) 950 dd larva. Transverse section of the thymus: cortex (a) and medulla (b).

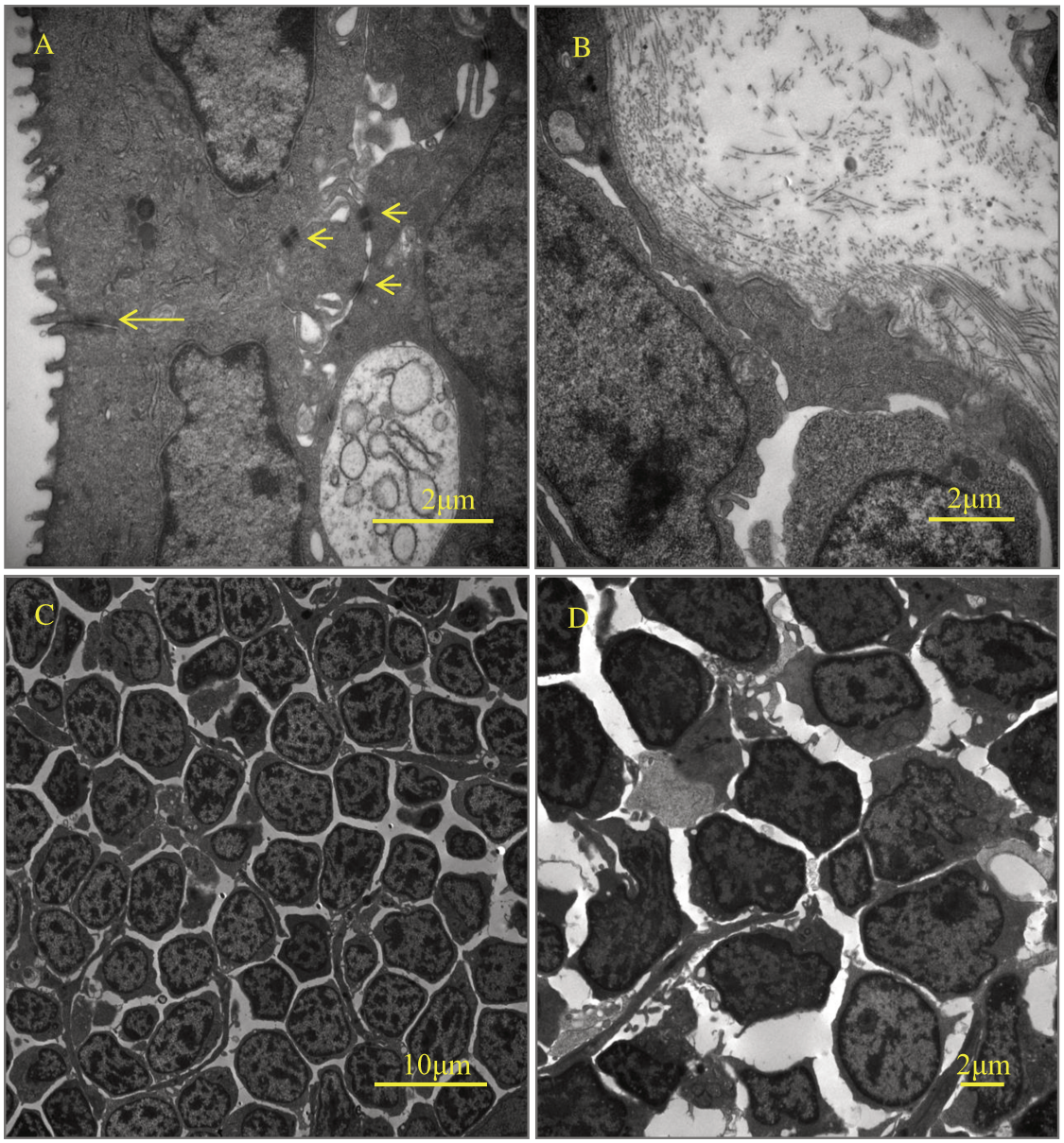

Figure 2.13. Transmission electron micrographs of A. oxyrinchus oxyrinchus thymus. A) Thymic epithelium in 768 growing degree-day (dd) larva, desmosomes (short arrows) and a tight junction (long arrow), B) area between cartilaginous skull and thymic epithelium in 768 dd larva, C) and D) thymocytes and epithelial reticular cells in 2013 dd fish.

Lymphocytes (%): There was no significant difference between the lymphocyte percentages in the different age groups (p-value=0.331).

Epithelial reticular cells (%): There was no significant difference between the reticular cell percentages in the different age groups (p-value=0.233).

Undifferentiated cells (%): There were significantly more undifferentiated cells in the older, larger fish (p-value=0.018).

Mitotic cells (%): No significant differences were present between the groups (p-value=0.701).

Apoptotic cells (%): No apoptotic cells were observed in the thymus sections analyzed.

Necrotic cells (%): No significant differences were present between the groups (p-value=0.212, respectively).

Table 2.4. Thymus cell percentages of different A. oxyrinchus oxyrinchus age groups. n=5; dd: growing degree-days; mean values ± standard errors of the mean (SEM).

Variables		768 dd	950 dd	1088 dd	1369 dd	2895 dd
Thymus	Erythrocytes/ thrombocytes (%)	0	0	0	0	0
	Heterophils (%)	0	0	0	1.0±1.0	0.1±0.1
	Lymphocytes (%)	0	90.3±1.2	91.5±1.5	77.3±15.9	72.3±6.9
	Eosinophils (%)	0	0.1±0.1	0	1.4±1.4	0
	Reticular cells (%)	0	5.1±0.8	4.2±1.1	15.8±10.4	11.3±2.9
	Undifferentiated cells (%)	0	1.7±0.6	3.8±0.8	2.8±1.8	13.6±5.2
	Mitotic cells (%)	0	0.6±0.3	0.2±0.1	0.6±0.5	1.6±1.2
	Necrotic cells (%)	0	2.2±0.8	0.4±0.3	0.6±0.5	1.6±0.9

2.5. Discussion

The first evidence of humoral and cellular immunity in salmonids is coincident with the onset of feeding[4]. In the current study involving sturgeon, the meningeal myeloid tissue, spleen and thymus were only evident well after this in 541 dd fish. This was approximately 400 dd after the onset of feeding at 154 dd. Although 541 dd sturgeon were within the size interval for which autologous IgM was first reported in salmonids (20-30mm)[9], no lymphocytes were present in any immune organs analyzed until 768 dd. The slow development of these immune organs may render sturgeon more vulnerable to viruses or other waterborne pathogens and may contribute to high mortalities seen in early life stages. Other factors such as maternal protective immunity and content of the yolk sac as well as the role of other relevant organs, particularly the gut, should also be considered in future developmental studies when assessing the overall immunity in sturgeon early life stages. The spiral valve plays perhaps an especially important immune role during the onset of feeding. The kidney was present in our samples in 165 dd larvae and possibly was present earlier than this sampling time. Both these organs were not analyzed in this study and should be studied further. In this study, the sturgeon mean total length increased with age except for the 950 dd group, which had the second highest mean total length. Other external factors such as more available food than in the other groups might explain this. However, this may also be due to the high size variability between fish of the same age group, the small sample size and an artifact of sampling.

The main lymphomyeloid tissues in sturgeon are the anterior part of the kidney, the meningeal myeloid tissue, the spleen, the thymus, the pericardial tissue and the spiral valve[15,16]. The kidney was not studied due to its friability and difficulty of sampling and

processing for TEM for all age groups but should be analyzed in future studies. Monocytes and macrophages were not counted in this study as a separate category and were only included in the "unknown/other cells" category. Monocytes are pro-phagocytic cells and in the tissues they turn into macrophages[20]. Using LM, shortnose sturgeon and Chinese sturgeon monocytes were described as large cells with abundant cytoplasm that was frequently vacuolated. The reniform or horseshoe-shaped nucleus was large, with prominent chromatin clumping and often a clearly visible nucleolus[18,19]. TEM analysis of peripheral blood of Chinese sturgeon identified the mature monocyte nucleus as having an eccentric position in the cell. The cytoplasm was filled with mitochondria and free ribosomes and the plasma membrane formed fine pseudopodia and blebs. These cells could be distinguished by the presence of large cytoplasmic vesicles of heterogeneous content corresponding to lysosomes. On LM and TEM, macrophage identification is based on the presence of cytoplasmic lysosomes containing digested material[18]. All of these morphological descriptions refer to mature monocytes or macrophages and not to those present in hemopoietic organs, where they are in different stages of development and maturation and surrounded by other cell types. Based on morphological parameters alone, consistent identification of monocytes and macrophages using TEM in the current study was not feasible across all age groups, as cells could only be identified when lysosomes and a nucleus were seen. In the future, studies similar to one done in carp to identify monocytes and macrophages in developing lymphoid tissue could be considered[21]. In that study, monoclonal antibodies against carp macrophages were used in flow cytometry, immunohistochemistry and immunoelectron microscopy techniques. Erythrocytes have an oval shape and an oval elongated nucleus. Thrombocytes have a spindle, pyriform or oval shape with the nucleus following the

cytoplasmic shape[19]. The high cell density of hemopoietic organs prevents an accurate differentiation of these cell types based on cell shape and nuclear features alone. The presence of cells in different stages of maturation in these organs makes nuclear chromatin characterization potentially difficult when used in cell classification. Therefore, accurate identification of thrombocytes and erythrocytes in the immune organs studied was not feasible and both cell types were grouped in one category.

The meningeal myeloid tissue was morphologically similar to that reported in previous studies[15] and it was first visible in H&E sections of 768 dd fish within the cranial cavity in a saddle-shape arrangement. This means that this tissue first appeared sometime between 541 and 768 dd of development at approximately 4 to 6 weeks post hatch. Also, considering that this tissue was only found in the 768 dd H&E samples, the fish length interval at which this tissue first appeared is likely between 1.8 and 1.9 cm. This range is based on the difference in average size between H&E and TEM samples for the time point. Regarding meningeal myeloid tissue cell populations and how they change over time, the lack of pattern in the percentages of the erythrocyte/thrombocyte group might be due to the random distribution of blood vessels and sinusesin individual sections cut for TEM examination. In this tissue, similar to what has been previously reported[12], size was more relevant in determining cell percentages than age, with the heterophil and eosinophil percentages being greater in the larger fish (950 and 2895 dd samples). Heterophils are neutrophils that lack myeloperoxidase (MPO) and have reduced ability for an oxidative burst[22]. The high percentages of heterophils found in meningeal myeloid tissues in the present study were consistent with it being described mainly as a granulopoietic tissue[15,16]. Lymphoid tissues are composed of a reticular cell framework that supports migratory and non-migratory cell populations and produces

reticular fibers[23]. Regarding the undifferentiated cells, less developed cell stages have a nucleus with a central region of euchromatin (uncondensed chromatin). Cell differentiation is accompanied by markedly increasing heterochromatin condensation in the peripheral nuclear region adjacent to the nuclear envelope[24]. The percentages of reticular cells and undifferentiated cells were significantly higher in the youngest animals (541 dd) compared to older ones, but this was mainly due to the fact that these were the only cell types present in the 541 dd specimens. This should therefore be taken into account when interpreting percentage results. The different percentages of undifferentiated cells across the age groups might also be related to the balance between the production, maturation and release of immune cells into circulation, but such aspects extend beyond the scope of this study.

The spleen serves as a blood reservoir and is a major secondary lymphoid organ in fish[15,16]. The spleen is located in the peritoneal cavity adjacent to the gut wall[25]. The histological appearance of the sturgeon spleen in the present study was consistent with that described in previous studies[15,16], with large arteries surrounded by large lymphoid follicles. In teleosts, lymphocytes are widely scattered and show only a slight tendency to accumulate around blood vessels and melanomacrophage centers (MMC). MMC are nodules or centers of pigment-containing cells (melanomacrophages), which occur in lymphohemopoietic tissues and/or the liver of most teleosts. No germinal centers are found in fish tissues[26,27]. The most relevant changes during splenic development in fish in the present study were in the heterophil percentages, which were significantly higher in the oldest fish when compared with the younger fish (2895 and 768 dd, respectively). Unlike that observed for meningeal myeloid tissue, fish size was not as relevant as age in affecting splenic cell type changes in this study. There were no significant changes in

lymphocyte percentages (absent in the youngest fish) but there was a significantly higher undifferentiated cell percentage in the younger fish compared to the oldest. This reflected a less developed spleen in the younger animals and progressive cell differentiation and maturation over time. Despite their size, the relatively high percentage of splenic undifferentiated cells in the 950 dd fish might indicate that they are less immunologically developed than the equivalently sized 2895 dd fish. This suggests that age might also play an important role in immunity at such early life stages. However, future studies are required to further assess the immune development in these early life stages.

The thymus is a key organ in the immune system of vertebrates[28]. In fish, it is a paired organ located in the dorsomedial aspect of the branchial cavity arising from primordia associated with the epithelium of the pharyngeal pouches[25]. The pharyngeal epithelium covering the thymus constitutes an effective barrier for the entry of both antigenic and non-antigenic materials from the pharyngeal cavity into the thymic parenchyma. This is accomplished by a continuous layer of epithelial cells with lateral intercellular spaces tightly sealed by intercellular junctions such as tight junctions[29]. The thymus in sturgeon is lobulated, divided into cortex and medulla and contains lymphocytes, reticular cells and macrophages, with no Hassall's corpuscles[15]. When the thymus was first visible in the samples examined in the present study (768 dd) and in the older age groups, the basic ultrastructure was similar to that described in other fish[23]. The epithelial reticular cells are supporting stromal cells present in the thymus; they are of epithelial origin and do not produce reticular fibers[20]. The desmosomes that join these reticular cells to each other can be used as a parameter for cell identification[30]. The predominant cell type in the thymus was the lymphocyte found in different stages of

maturation. The only significant difference found was in undifferentiated cell percentages (highest in the oldest group), which might reflect a more active and proliferative thymus in the older animals. However, there were no significant differences in the percentages of mitotic and apoptotic cells between the different groups (even though they were higher in the oldest age group). Since a higher percentage of mitotic cells[31] would be present in more proliferative tissues, a significant difference between the groups might have indicated a more active thymus. In the thymus, apoptosis of immature T cells is an important mechanism, which produces clonal deletion and consequently self-tolerance, essential for an adequate T cell function and immune response[32]. Apoptotic cells show membrane blebbing, nuclear shrinkage and chromatin condensation, a reduction in cell volume and the formation of apoptotic bodies. In contrast, necrotic cells show both nuclear and cytoplasmic swelling with cell membrane and nuclear membrane dissolution or lysis[33, 34]. No apoptotic cells were found in any of the thymus sections analyzed. However, considering that in sea bass (Dicentrarchus labrax), no apoptotic cells were detected at around 432 dd and at approximately 1184 dd only 47±8 apoptotic cells were found per 100,000 cells[35], it is not surprising that no apoptotic cells were found in the 4 thymus sections analyzed per fish in the present study. Regarding the necrotic cells, they can occur due to numerous reasons such as stress, heat and osmotic shock, among others[36]. Even though no significant differences were observed between the groups, further research would be needed to assess overall functional activity in the thymus of sturgeon early life stages.

The unknown cells/granulocytes observed in the present stydy have not been described in the blood of Chinese sturgeon[18]. The first granulocyte could be a stage of heterophil differentiation and maturation, a mast cell (eosinophilic granular cell) or yet

another cell type such as a basophil[18]. There were not enough cells with crystalline structures observed to conclude that this is a specific cell type; further research is necessary to adequately classify these cell types in sturgeon.

It is relevant to emphasize that, unlike teleosts, an effective immune response may not occur in sturgeon larvae at the onset of feeding. This is supported by the later appearance of the thymus in sturgeon, despite them having a similar size interval to that of salmonids when autologous IgM is first produced[9]. However, further research is needed to assess the immune development of these and other immune organs (kidney and spiral valve) in Atlantic sturgeon juveniles and following pathogen challenge or immunostimulation.

2.6. Conclusions

The morphology of these immune organs and cell types was similar to that previously described in other sturgeon species[15,16,18,19]. This ontogeny study demonstrates that these immune organs were first visible at the light microscopic level between 541 dd (spleen) and 768 dd fish (meningeal myeloid tissue and thymus), approximately 400 dd after the onset of feeding. With the exception of an uncategorized granulocyte found in low numbers, all cell types observed were similar to that of other fish species. Heterophils significantly increased with fish size in the meningeal myeloid tissue and mostly with age in the spleen. The thymus was mainly composed of lymphocytes. It is relevant to emphasize that while autologous IgM has been detected in salmon as early as 540 dd[9], no lymphocytes were observed in Atlantic sturgeon in any of the immune organs analyzed until 768 dd. The ultrastructure of the thymus was similar across the different age groups from the 768 dd group onward. Overall, younger animals

had a higher percentage of undifferentiated cells than the older groups, except for within the thymic tissue, where they were significantly higher in the older group. This may be due to a more active and proliferative thymus in these fish, which might reflect a more functional thymus in the later age group. Regarding the unknown granulocyte described, further morphological descriptions are necessary to adequately classify it.

In summary, clear ultrastructural organization of these immune organs was only observed in samples older than 541 dd (33 dph) and an effective immune response might not occur in the early life stages prior and during the onset of feeding. This study of sturgeon immune organ characterization during development is useful in suggesting the potential onset of functional lymphocyte and adaptive responses. However, further research is needed to assess the immune development of these and other immune organs (kidney and spiral valve) and overall immune competence in these life stages, which may be influenced by other factors such as the presence of immune factors transferred from the mother. Further research is also needed to assess immune development in Atlantic sturgeon juveniles and following pathogen challenge or immunostimulation.

2.7. References

1. Bronzi, P., Rosenthal, H., & Gessner, J. 2011. Global sturgeon aquaculture production: An overview. J. Appl. Ichthyol. 27, 169-175.

2. Wang, Z. & Zhang, S. 2010. The role of lysozyme and complement in the antibacterial activity of zebrafish (Danio rerio) egg cytosol. Fish Shellfish Immunol. 29, 773-777.

3. Kim, B., Nam, B., Kim, J., Park, H., Song, J. & Park, C. 2011. Molecular characterization and expression analysis of a fish-egg lectin in rock bream, and its response to bacterial or viral infection. Fish Shellfish Immunol. 31, 1201-1207.

4. Olsen, Y.A. & Press, C.M. 1997. Degradation kinetics of immunoglobulin in the egg, alevin and fry of Atlantic salmon, Salmo salar L., and the localization of immunoglobulin in the egg. Fish Shellfish Immunol. 7, 81-91.

5. Swain, P., Dash, S., Bal, J., Routray, P., Sahoo, P.K., Sahoo, S.K., Saurab, S., Gupta, S.D. & Meher, P.K. 2006. Passive transfer of maternal antibodies and their existence in eggs, larvae and fry of Indian major carp, Labeo rohita (Ham.). Fish Shellfish Immunol. 20, 519-527.

6. Mohler, J.W., King, M.K. & Farrell, P.R. 2000. Growth and survival of first-feeding and fingerling Atlantic sturgeon under culture conditions. N. Am. J. Aquacult. 62, 174-183.

7. Conte, F.S., Doroshov, S.I., Lutes, P.B. & Strange, E.M. 1988. Hatchery manual for white sturgeon, Acipenser transmontanus R., with application to other North American Acipenseridae. Oakland: Div. Agric. Nat. Res., University of California. Pp. 67.

8. Saurabh, S. & Sahoo, P.K. 2008. Lysozyme: an important defense molecule of fish innate immune system. Aquacult. Res. 39, 223-239.

9. Magnadottir, B., Lange, B.S., Gudmundsdottir, S., Bøgwald, J. & Dalmo, R.A. 2005. Ontogeny of humoral immune parameters in fish. Fish Shellfish Immunol. 19, 429-439.

10. Petrie-Hanson, L. & Ainsworth, A.J. 2000. Differential cytochemical staining characteristics of channel catfish leukocytes identify cell populations in lymphoid organs. Vet. Immunol. Immunopathol. 73, 129-144.

11. Tatner, M.F. & Manning, M. 1983. Growth of the lymphoid organs in rainbow trout, Salmo gairdneri from one to fifteen months of age. J. Zool. 199, 503-520.

12. Johnson, K.A., Flynn, J.K. & Amend, D.F. 1982. Onset of immunity in salmonid fry vaccinated by direct immersion in Vibrio anguillarum and Yersinia ruckeri bacterins.

J. Fish Dis. 5, 197-205.

13. Neuheimer, A.B. & Taggart, C.T. 2007. The growing degree-day and fish size-at-age: the overlooked metric. Can. J. Fish. Aquat. Sci. 64, 375-385.

14. Bancroft, J.D. & Cook, H.C. 1995. Manual of Histological Techniques and their diagnostic application. Churchill Livingstone, Singapore. Pp. 27.

15. Fänge, R. 1986. Lymphoid organs in sturgeons (Acipenseridae). Vet. Immunol. Immunopathol. 12, 153-161.

16. Lange, M.A., Govyadinova, A.A. & Khrushchev, N.G. 2000. Study on localization of hemopoietic tissue in sturgeon. Sov. J. Dev. Biol. 31, 372-376.

17. Scharrer, E. 1944. The histology of the meningeal myeloid tissue in the ganoids Amia and Lepisosteus. Anat. Rec. 88, 291-310.

18. Zexia G., Weimin, W., Yi, Y., Abbas, K., Dapeng, L., Guiwei, Z. & Diana, J. 2007. Morphological studies of peripheral blood cells of the Chinese sturgeon, Acipenser sinensis. Fish Physiol. Biochem. 33, 213-222.

19. Knowles, S., Hrubec, T.C., Smith, S.A. & Bakal, R.S. 2006. Hematology and plasma chemistry reference intervals for cultured shortnose sturgeon (Acipenser brevirostrum). Vet. Clin. Pathol. 35, 434-440.

20. Eurell, J.A. & Frappier, B.L. 2006. Dellman's Textbook of Veterinary Histology. 6th edition, Blackwell Publishing, Iowa, USA. Pp. 61-66, 70-75, 136,137, 140, 141, 147-152.

21. Romano, N., Picchietti, S., Taverne-Thiele, J.J., Taverne, N., Abelli, L., Mastrolia, L., Verburg-van Kemenade, B.M.L. & Rombout, J.H.W.M. 1998. Distribution of macrophages during fish development: an immunohistochemical study in carp (Cyprinus carpio, L.). Anat. Embryol. 198, 31-41.

22. Palić, D., Beck, L.S., Palić, J. & Andreasen, C.B. 2011. Use of rapid cytochemical staining to characterize fish blood granulocytes in species of special concern and determine potential for function testing. Fish Shellfish Immunol. 30, 646-652.

23. Press, A. McL. & Evensen, O. 1999. The morphology of the immune system in teleost fishes. Fish Shellfish Immunol. 9, 309-318.

24. Smetana, K., Mikulenková, D. & Klamová, H. 2011. Heterochromatin density (condensation) during cell differentiation and maturation using the human granulocytic lineage of chronic myeloid leukaemia as a convenient model. Folia Biol. (Praha). 57, 216-221.

25. Roberts, R. 1989. Fish Pathology. 2nd edition, Bailliere Tindall, Great Britain. Pp.

31-32.

26. Zapata, A. 1982. Lymphoid organs of teleost fish. III. Splenic lymphoid tissue of Rutilus rutilus and Gobio gobio. Dev. Comp. Immunol. 6, 87-94.

27. Fänge, R & Nilsson, S. 1985. The fish spleen: structure and function. Experientia. 41, 152-158.

28. Flajnik, M., 2002. Comparative analyses of immunoglobulin genes: surprises and portents. Nat. Rev. Immunol. 2, 688-698.

29. Castillo, A., Razquin, B., Villena, A.J., Zapata, A.G. & López-Fierro, P. 1998. Thymic barriers to antigen entry during the post-hatching development of the thymus of rainbow trout, Oncorhynchus mykiss. Fish Shellfish Immunol. 8, 157-170.

30. Savino, W. & Santa-Rosa, G.L. 1982. Review: Histophysiology of thymic epithelial reticular cells. Arch. Histol. Jpn. 45, 139-144.

31. Robbins, E. & Gonatas, N. 1964. The ultrastructure of a mammalian cell during the mitotic cycle. J. Cell Biol.

32. Mandel, T. 1970. Differentiation of epithelial cells in the mouse thymus. Dev. Comp. Immunol. 106, 498-515.

33. Smith, C.A., Williams, G.T., Kingston, R., Jenkinson, E.J. & Owen, J.J.T. 1989. Antibodies to CD3/T-cell receptor complex induce death by apoptosis in immature T cells in thymic cultures. Nature. 337, 181-184.

34. Allen, R.T., Hunter III, W.J. & Agrawal, D.K. 1997. Morphological and biochemical characterization and analysis of apoptosis. J. Pharmacol. Toxicol. Methods. 37, 215-228.

35. Abelli, L., Baldassini, M.R., Meschini, R. & Mastrolia, L. 1998. Apoptosis of thymocytes in developing sea bass Dicentrarchus labrax (L.). Fish Shellfish Immunol. 8, 13-24.

36. Krysko, D. V., Berghe, T. V., D'Herde, K. & Vandenabeele, P. 2008. Apoptosis and necrosis: Detection, discrimination and phagocytosis. Methods. 44, 205–221.

Chapter 3.0 Temperature effects on immunological development and responsiveness in Acipenserid juveniles

3.1. Abstract

Sturgeon are an important evolutionary taxa of which little is known regarding their responses to environmental factors. Water temperature strongly influences growth in fish; however, its effect on sturgeon immune responses is unknown. The objective of this study was to assess how temperature affects immune responses in shortnose sturgeon (Acipenser brevirostrum) relevant immune organs such as the meningeal myeloid tissue, spleen, thymus and skin. These responses were studied in 2 different sizes of same age juvenile sturgeon kept at either 11°C or 20°C (4 treatment groups), before and after exposure to an ectoparasitic copepod (Dichelesthium oblongum). Based on a differential cell count, temperature was found to strongly influence immune cell production in the meningeal myeloid tissue, regardless of the fish sizes considered. Morphometric analysis of splenic white pulp showed a transient response to temperature. There were no differences between the groups in the morphometric analysis of thymus size, although further research in this area is warranted. Interferon regulatory factors 1 and 2 (IRF-1 and IRF-2) and matrix metalloprotease 9 (MMP-9) gene expression were analyzed in the spleen and skin. Splenic IRF-1 and IRF-2 had a similar expression profile and were significantly higher in fish kept at 20°C for the first 6 weeks of the study but not by 14 weeks. In the skin, IRF-1 was significantly higher in the fish kept at 11°C over the first 6 weeks of the study. IRF-2 had a similar profile but there were no differences between the groups by the end of the trial. The parasite exposure was likely unsuccessful, which is consistent with the lack of changes in the MMP-9 expression in the skin for the different groups. However, the maintenance of

adult female parasites and copepods was attained for the first time in an experimental setting. In conclusion, higher water temperatures (up to 20°C) may have beneficial effects in maximizing growth and improving immunological capacity, regardless of the fish sizes considered in this study. This information could be important in decreasing early life stage mortalities in shortnose sturgeon culture.

Key words: Sturgeon, immunity, temperature, size.

3.2. Introduction

Sturgeon are harvested for their meat and eggs (sold worldwide as caviar) and sturgeon aquaculture has increased considerably in the last two decades. These phylogenetically primitive fish are among the most economically important[1] and they are considered to be a transition between major taxa, thus useful in the understanding of vertebrate evolution. Little is known about immunological development and response to environmental factors in sturgeon. A better understanding of these interactions is important to optimize aquaculture rearing conditions and potentially aid wildlife conservation programs.

Life history and migration patterns expose sturgeon to significant temperature fluctuations[2], with temperature tolerance increasing with age and size. Temperature influences many of the behaviors and activities of sturgeon, including spawning, egg development and seasonal movements[3]. Aside from the consideration of the minimum size at which fish are thought to develop a functional immune response, water temperature is believed to be the single most important factor in development and is known to influence all physiological functions in fish[4]. Many studies have focused on

how environmental temperatures affect the immune system in teleosts. An overall decreased immune response at lower compared to higher water temperatures has been reported for different fish species[5] and fish reared at lower temperatures rely more heavily on innate immunity[6,7]. Both cellular and humoral specific immune responses are temperature dependent in fish[8,9]. These defense mechanisms are shown to be suppressed by temperatures averaging 10°C below the host optimal growth temperature[10]. No such studies have been done for shortnose sturgeon (Acipenser brevirostrum), but data suggests they prefer and perform optimally under 25°C conditions[11].

Size also influences functional immune responses in fish. Research on salmonids supports a stronger and more developed immunological response in larger fish within a group of animals of the same age[12]. Although studies on juvenile shortnose sturgeon show rapid growth of 14-30 cm during the first years at river temperatures ranging from 9°C to 15°C[11], immunological development and competence over this time is unknown. Culture of white sturgeon, A. transmontanus[13-15] and other sturgeon species[16] has been hampered by viral infections of juvenile fish. Despite evidence of strong antibody responses against viruses in white sturgeon[17], a lack of basic understanding of innate and or specific antiviral responses in sturgeon prevents effective minimization of the impact such pathogens have on sturgeon aquaculture and conservation efforts. Multiple studies[14,16] have described stocking density and husbandry practices in multiple sturgeon species as a risk factor for mortality and viral skin infections, but it is not known how environmental conditions affect leucocyte populations, antiviral mechanisms and skin healing in sturgeon.

The objective of this study was to assess how temperature influences the development and responsiveness of immune organs in shortnose sturgeon, focusing on

meningeal myeloid tissue, spleen, thymus and skin. In order to characterize these organs, both morphological and immune gene expression analysis were performed in 4 groups of 1-year old fish divided according to size and temperature (smaller and larger fish kept at either 11°C or 20°C). As a measure of immunocompetence, the fish were exposed to a common sturgeon ectoparasitic copepod (Dichelesthium oblongum). Given that temperature influences immune responses in fish[4], our hypothesis was that the fish kept at 11°C should rely more heavily on innate immunity (with a higher production of innate immune cells) when compared to the corresponding fish kept at 20°C, regardless of size. Since a stronger immunological response has been observed in larger fish within a group of animals of the same age, it was also hypothesized that the larger animals would have a more responsive immune system when compared to the smaller fish at a given temperature.

3.3. Materials and methods

3.3.1. Fish husbandry and parasite exposure

All experimental protocols followed the guidelines given in 2005 by the Canadian Council on Animal Care (http://www.ccac.ca/Documents/Standards/Guidelines/Fish.pdf) and were approved by the UPEI Animal Care Committee.

Fish: In April of 2012, 240 five to twenty-gram 7- month-old shortnose sturgeon were transported from Acadian Sturgeon and Caviar Inc. (Carter's Point, New Brunswick, Canada) to the Aquatic Animal Facility (AAF) of the Atlantic Veterinary College (AVC), UPEI. After a 2-week acclimation period at 11°C, the fish were divided in 2 groups according to size and distributed among eight 150L freshwater flow-through

circular tanks, with 30 fish per tank and 12 hour light/dark circadian cycles. The temperature in four tanks was increased to 20°C at a rate of 2°C/day[18] and four other tanks were kept at 10.8±0.07°C (mean±SD, measured weekly throughout the trial). After the fish were acclimated to the tanks at either of the temperatures, two replicate tanks were assigned per treatment (smaller or larger sturgeon, at each of the two temperatures). The fish were fed a regular sturgeon diet (2mm pellet Corey diet, initially 1% and then increased to 2.5% body weight/day) divided over 2 feeds. Water quality parameters such as concentrations of ammonia, nitrites, nitrates and dissolved oxygen (g/L) as well as pH and total gas saturation were checked regularly. Ten weeks after the beginning of the temperature trial, two tanks from each temperature group were exposed to adult D. oblongum as described in the next section. One week later the fish were checked for attached parasites, re-exposed to D. oblongum copepodids and the fish re-checked 3 weeks later.

Parasites: D. oblongum adults were randomly collected from wild sturgeon in the Saint John River, NB, and kept at the AVC in freshwater for 24 hours until placed in the experimental tanks (7 adult female parasites per tank). Flow was kept off for up to 5 minutes in each tank during exposure until all female D. oblongum had been taken up by the fish. Egg strings from adult female parasites were also collected and hatched in a seawater hatchery system, with 33-36 parts per thousand of seawater salt concentration, at 11±1^{o}C (mean±SD)[19]. The salinity tolerance of this parasite is unknown, but while adult females survive on hosts for over 1 month in fresh water, development of copepodid stages in the laboratory was inhibited below 20 parts per thousand in previous experiments (M. Fast, personal observation). Therefore, egg strings were maintained in saltwater for 1 week until development into copepodid stages was achieved. They were

then transferred to fresh water for 2 days to ensure their survival at a low salinity prior to addition to the fish tanks (50 copepodids per tank). In summary, 7 female adult parasites and 50 copepodids were introduced in each of the "parasite-exposed" tanks.

3.3.2. Sample collection

The fish were kept off feed 24 hours prior to each sampling day to allow for their guts to be emptied before sampling. This reduces stress and improves water quality at the time of sampling and minimizes contamination of tissues by gut content upon sample collection. After the initial 2 week acclimation period, two fish per tank were sampled for reference and euthanized with 0.2g/L of tricaine methanesulfonate (TMS). The spleen and skin samples (dorsocaudal to the pectoral fin and ventral to the lateral row of scutes) were collected from one fish per tank, placed in Trizol Reagent (TRI Reagent), homogenized and stored at -80°C until further gene expression analysis. The head (containing the thymus and meningeal myeloid tissue) and spleen were collected from the second fish and placed in 10% neutral buffered formalin (NBF) for light microscopy (LM). The animals were sampled at 3, 6 and 14 weeks post temperature increase (time points 1, 2 and 3, respectively and the same tissues were sampled as for the reference point): 3 fish per tank for LM and 3 fish per tank for gene expression analysis. Blood was collected to determine packed cell volume (PCV) from the fish sampled for LM at 4 weeks after time point 2 (10 weeks post temperature change) and at 14 weeks (Appendix C - Figure C.1).

3.3.3. Sample processing

Light Microscopy (LM): Formalin-fixed head and spleen samples were trimmed, dehydrated in a graded series of ethanols to xylene, embedded in paraffin, cut in 5µm thick sections and stained with hematoxylin and eosin (H&E)[20]. Serial sections were cut for a subset of the fish (all 6 small fish kept at 11°C at time 3) and stained with H&E for morphometric standardization of the thymus.

Gene Expression Analysis: Total RNA was extracted from all spleen and skin samples. Approximately 50 mg of tissue was added to 1.5 mL of TRI Reagent[21] and mechanically macerated with a homogenizer (VWR, Mississauga, ON). For sturgeon spleen, we previously observed poor quality RNA despite rapid euthanasia and fish sampling. Therefore, all spleens sampled for gene expression were collected immediately following euthanasia of the fish and homogenized in TRI Reagent within a few minutes. Macerated tissue in TRI Reagent was then frozen at -80°C until further RNA extraction and processing. Skin samples were collected on dry ice and frozen at -80°C shortly thereafter, and homogenization in TRI Reagent done at a later date. Following a 5 minute incubation period at room temperature (RT; 22°C) in TRI Reagent, 300 µL of chloroform (BDH, West Chester, PA) were added to each homogenized sample. Tubes were capped and shaken vigorously for 15 seconds. Following a 3 minute RT incubation, the samples were centrifuged at 10,000xg for 15 minutes at 4°C. The aqueous phase was transferred to a new 1.5 mL tube and an equal volume of molecular grade isopropyl alcohol (Sigma-Aldrich, St. Louis, MO) was added to each tube. The tubes were mixed by inversion 3 times and incubated for 10 minutes at RT. The RNA was pelleted by centrifugation at 10,000xg (10 minutes at 4°C). The pellets were then washed with 750 µL of ice cold 75% molecular grade ethanol (BDH)

and centrifuged at 7500xg for 5 minutes at 4°C. RNA pellets were air dried for approximately 5 minutes. The pellets were resuspended in 100-200µL of molecular grade water before quantification on a NanoDrop-2000 spectrophotometer (Thermo Scientific, Ottawa, ON). Samples were stored at -80°C until DNase treatment. 5µg of isolated RNA were DNase treated using a TURBO DNase freeTM kit (Ambion, Foster City, CA). The procedure was carried out according to the manufacturer instructions. A random subset of RNA from each tissue was analyzed by Experion (Bio-Rad, Hercules, CA) and all RNA integrity numbers (RINs) were > 6.5. cDNA synthesis was performed on 1µg of DNase treated total RNA using a Reverse Transcription System (Biorad, Hercules, California), according to the manufacturer instructions. cDNA was stored at -20°C until use for qPCR. Primer sets were obtained for 3 reference genes (EF-1, β-actin and 18S) and 3 genes of interest (IRF-1, IRF-2 and matrix metalloprotease 9 (MMP-9). Degenerate primers were designed from Acipenseridae sequences when available and supplemented with sequences from Atlantic salmon (Salmo salar), rainbow trout (Oncorhynchus mykiss), Atlantic cod (Gadus morhua) and zebrafish (Danio rerio) (F. Clark, personal communication). Sequences were obtained from these degenerate primers using shortnose sturgeon (A. brevirostrum) spleen cDNA, which were then used to generate qPCR primers using Primer3 software (optimized by S. Purcell) (Table 1). The qPCR products were cloned (pGem®-T Easy vector, Thermo Scientific) and sequenced (Macrogen Inc., Rockville, MD) to confirm their identity. Each qPCR product was run in a 96-well plate (Eppendorf, Mississauga, ON) combining 0.5µL of forward primer (10µM), 0.5µL (10µM) of reverse primer, 5µL of 2x GoTaq® qPCR Master Mix (Biorad), 4µL of nuclease free water (Lonza, Rockland, ME) and 1µL of cDNA. qPCRs were performed using a Realplex thermocycler (Eppendorf) under the

following conditions: for genes EF-1, β-actin, 18s and IRF-2, the initial denaturation of 2 minutes at 95 °C, followed by 40 amplification cycles of 95 °C for 10 seconds and annealing for 30 seconds at 54.5 °C. At the end of each qPCR, a melt curve analysis was performed to ensure that only one product was amplified. The annealing temperatures for IRF-1 and MMP-9 were 55.6°C and 57.3°C, respectively.

Efficiencies of the qPCR reactions were determined for each gene and tissue type, using a series (5) of 1:5 dilutions of pooled spleen or skin cDNA (Table 3.1). These were run in triplicate wells for a minimum of three times each to ensure consistency across runs (r^2 greater than 0.95 in all cases). An internal positive control was also included on each plate to ensure inter-plate consistency. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method, where a mean reference value was obtained using the reference genes for normalization[22]. All reference genes passed geNORM requirements for stability on both tissue types to be included for analysis ($M < 0.5$, $CV < 0.3$).

3.3.4. Histological analysis

3.3.4.1. Meningeal myeloid tissue

500 cells per fish were manually counted (magnification: oil, 1000x) and a differential cell count and percentage of the total cells was calculated for each cell type. The following cell categories were considered based on previous studies done in meningeal myeloid tissue[23,24]: erythrocytes and erythrocyte precursors, thrombocytes, blast cells, lymphocytes, eosinophils, myeloid progenitor cells (granulocyte precursor cells) and immature and mature heterophils. Sturgeon neutrophils lack peroxidases, defining them as heterophils[25]. Unidentified cells were also counted.

Table 3.1. Shortnose sturgeon (A. brevirostrum, Ab) genes used for spleen and skin samples, forward and reverse primer sequences and corresponding Standard Curve Efficiencies (SCE).

Gene	Primer	Sequence	SCE Spleen	SCE Skin
Ab β-actin	Forward	CAT TGT CAC CAA CTG GGA TGA C	0.94	0.90
	Reverse	ACA CGC AGC TCA TTG TAG AAG GT		
Ab 18S	Forward	GAT GCC GAC TGG CGA TCC GG	0.91	1.04
	Reverse	GCC ATG CAC CAC CAC CCA CA		
Ab EF1a	Forward	GGA CTC CAC TGA GCC ACC T	0.96	0.94
	Reverse	GGG TTG TAG CCG ATC TTC TTG		
Ab IRF-1	Forward	AGTTGGCCTTCCATGTTTTC	0.93	1.04
	Reverse	GTGGGCCCTACACACAGG		
Ab IRF-2(9)	Forward	TTTTCAGATTCCCTGGATGC	1	1.01
	Reverse	TGTGGATGGCCCAGTTTC		
Ab MMP-9	Forward	CTTCAGGATGTCGTACTTCACG	0.94	1.01
	Reverse	TTTGCCAGGACCGTTTCTAC		

3.3.4.2. Spleen and thymus

The image analysis software Bioquant® was used for morphometric analysis of the spleen and thymus using images of the H&E stained LM sections for both tissues. For each fish, 3 images of the spleen were taken based on organ size (magnification: 100x) and the total spleen area and white pulp area measured accordingly (μm^2). The white pulp percentage was obtained for each image and the values averaged for statistical analysis. For the thymus, the area of each bilateral organ was measured for every fish (including the serial sections of the small fish kept at 11°C in time 3) and averaged (μm^2), as well as the areas of the brain and the notochord present in the corresponding section. In order to compare the thymus areas between the different groups, the within-fish proportion of variation and the between-fish proportion of variation were determined. The latter is the intracluster correlation coefficient or reliability coefficient. In addition, in order to compare sections between different fish and different groups and considering that the areas measured per fish pertain to only one cut section of the thymus (cut section varies between fish), the measurements were adjusted to a reference organ. Specifically, correlations were done using the brain or notochord also present in the slides.

3.3.5. Statistical analysis

Minitab 16® was used for all statistical analyses. General Linear Models were used to compare the different groups and assess the interactions between temperature and size on the different tissues over time. The Bonferroni method was used for the multiple comparisons tested for the LM and gene expression analysis, with a cut-off of $p<0.05$ for statistical significance (Appendix B - Tables B.2-B.4). Unless specified, all

values are reported as means ± standard errors (SE).

3.4. Results

3.4.1. Light microscopy

Histological sections (H&E) of the head containing meningeal myeloid tissue (Figures 3.1A and 3.1B) and thymus (Figures 3.1A and 3.1C), and the spleen (Figure 3.1D) were analyzed for all fish groups. There were no tank effects for any of the variables studied and the data from replicate tanks was pooled for each group.

All p-values are listed in Appendix tables B.2-B.4. Growth and the variables for the meningeal myeloid tissue and the spleen are listed in Table 3.2. At 3 weeks post temperature change, the larger fish were significantly heavier than the smaller fish for both temperatures (p-value<0.005 for both) (Figure 3.2). The larger fish at 20°C were also significantly heavier than the smaller fish at 11°C (p-value<0.005 for all time points). During the trial, only the smaller fish housed at 20°C grew significantly over time (p-value<0.005), catching up with the larger fish in size by the end of the trial (14 weeks post temperature change).

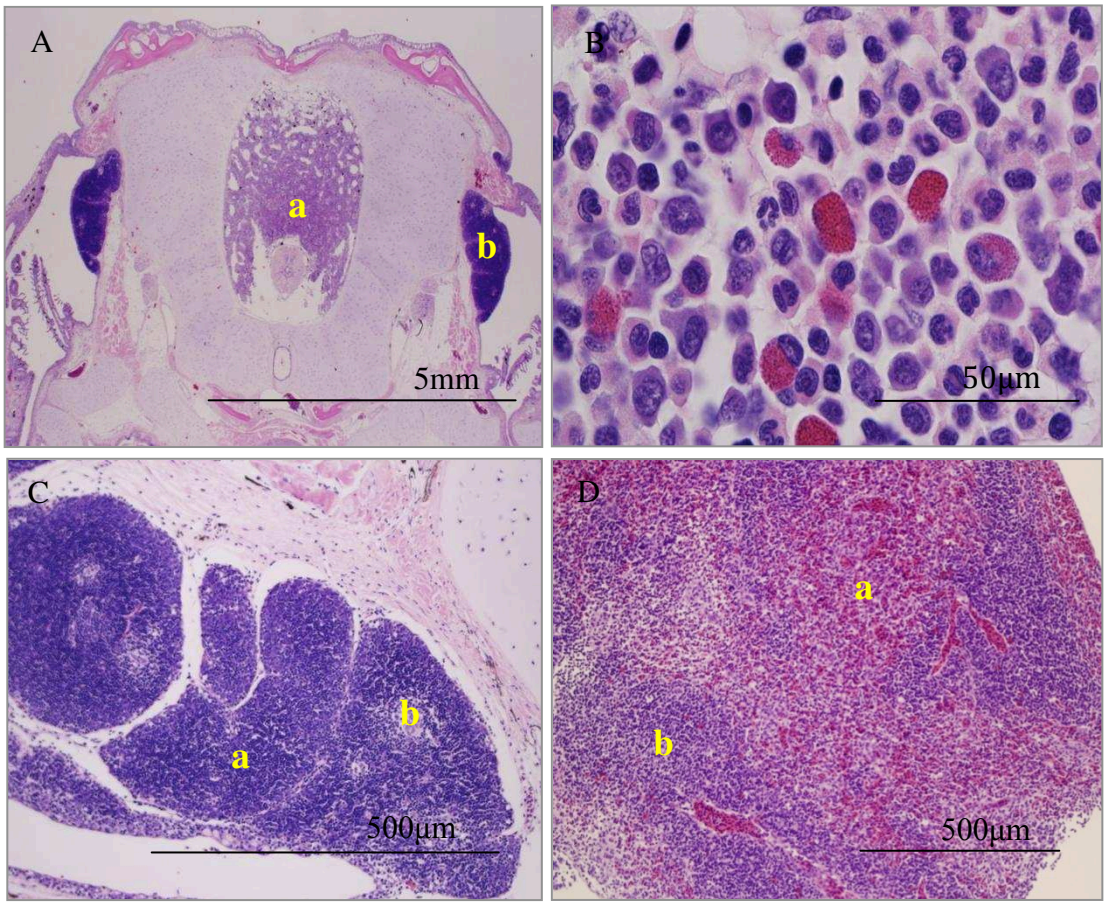

Figure 3.1. Juvenile A. brevirostrum, Hematoxylin & Eosin. A) Transverse section of head with meningeal myeloid tissue (a) and bilateral thymus (b), B) meningeal myeloid tissue, C) Thymus with cortex (a) and less densely packed medulla (b), D) splenic red pulp (a) and white pulp (b).

Table 3.2. Meningeal myeloid tissue and splenic variables of different sized A. brevirostrum held at 11°C/20°C. n=6; mean values ± standard errors of the mean (SEM).

Variables		Time Point	Small Fish		Large Fish	
			11°C	20°C	11°C	20°C
Weight (g)		0	4.68±1.89		15.64±4.34	
		1	7.32±0.58	9.08±1.56	20.50±1.80	24.38±2.86
		2	7.12±1.57	13.07±2.31	18.67±1.76	36.92±7.96
		3	11.10±1.46	45.15±7.46	24.17±2.68	33.18±5.88
Meningeal Myeloid Tissue	Erythrocytes and their precursors (%)	0	4.4±3.40		1.15±1.37	
		1	5.0±1.26	3.0±1.30	10.07±1.85	11.93±2.74
		2	13.2±4.3	6.03±2.49	9.83±3.7	7.97±1.58
		3	2.90±0.6	10.63±2.69	8.47±1.65	12.0±1.90
	Thrombocytes (%)	0	1.55±0.62		0.9±1.09	
		1	2.12±0.51	2.80±0.96	5.20±0.96	9.17±2.16
		2	0.56±0.12	6.97±1.99	5.27±2.07	9.43±1.05
		3	0.13±0.07	1.97±0.42	0.47±0.24	1.37±0.40
	Blast cells (%)	0	0.3±0.26		7.35±4.67	
		1	2.36±0.64	0.68±0.35	0.87±0.83	0.67±0.41
		2	1.96±1.06	0.03±0.03	0.1±0.07	0
		3	0.50±0.20	0.40±0.16	1.07±0.54	0
	Myeloid progenitor cells (%)	0	35.55±17.32		27.5±7.66	
		1	51.84±6.36	13.8±1.89	39.8±5.81	17.20±2.98
		2	32.36±6.06	21.77±3.41	40.13±4.79	23.57±3.67
		3	29.73±5.98	8.77±2.57	47.2±4.21	6.13±0.94
	Immature Heterophils (%)	0	42.8±12.81		35.5±4.39	
		1	28.36±5.76	32.92±3.76	26.43±7.43	22.0±3.69
		2	22.52±2.31	34.40±4.06	33.30±3.16	26.07±3.31
		3	43.47±4.37	26.37±4.40	27.70±3.29	20.70±2.02
	Heterophils (%)	0	6.35±9.68		9.45±2.43	
		1	1.52±0.80	14.12±2.34	4.0±1.64	8.23±2.90
		2	1.44±0.92	4.5±1.85	1.07±0.44	9.17±1.43
		3	13.07±3.77	19.77±1.88	4.50±1.37	33.50±6.59
	Lymphocytes (%)	0	3.2±3.17		4.1±2.64	
		1	3.16±1.46	14.36±4.48	3.0±1.14	16.23±1.98
		2	4.60±1.11	13.50±3.56	3.53±0.96	12.90±1.26
		3	4.30±0.80	18.07±4.61	3.83±0.47	9.37±3.08
	Eosinophils (%)	0	2.05±2.07		5.4±3.47	
		1	3.76±0.98	10.56±2.06	6.4±1.22	7.57±2.38
		2	3.24±1.21	8.57±3.68	2.4±0.18	8.13±1.50
		3	2.57±0.71	8.33±1.14	3.83±0.58	8.93±2.08
	Unclassified cells (%)	0	3.8±1.61		8.65±3.19	
		1	1.88±0.75	7.76±0.66	4.23±1.41	7.0±1.67
		2	9.0±1.51	4.23±1.04	4.37±0.87	3.67±0.78
		3	3.43±0.61	5.60±1.72	2.93±0.99	3.83±1.22
Spleen	White pulp (%)	0	17.35±0.03		16.44±0.04	
		1	17.46±2.30	40.94±6.34	27.56±2.23	29.9±1.67
		2	20.93±2.27	29.21±2.77	18.86±2.38	28.23±2.35
		3	26.06±1.98	25.54±2.99	25.47±1.17	23.86±1.84

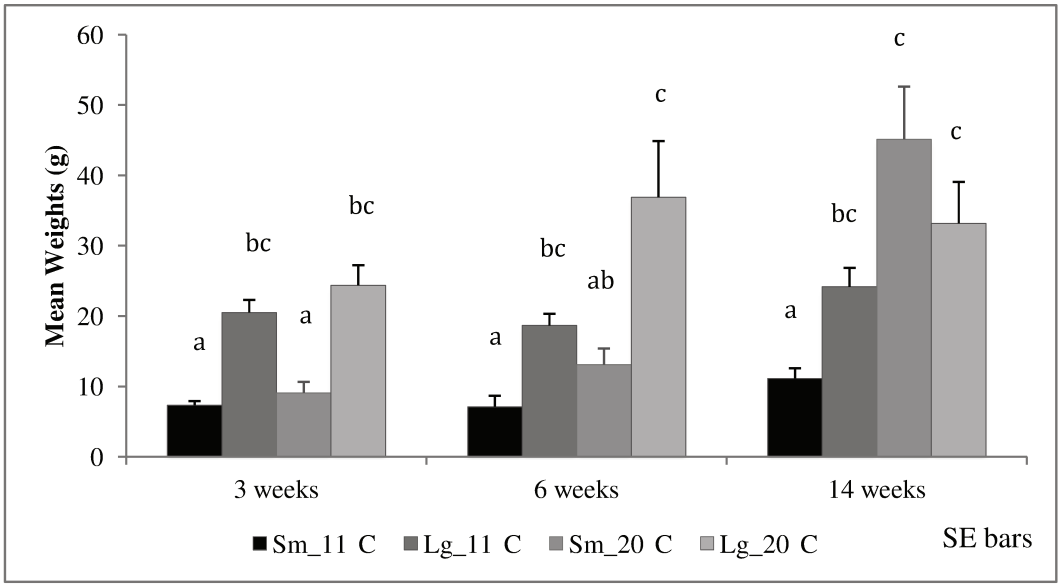

Figure 3.2. Growth of the different groups of A. brevirostrum (smaller and larger sizes held at 11°C and 20°C). n=6; different letters signify statistically significant differences between groups.

3.4.1.1. Meningeal myeloid tissue:

The cell percentages were as follows (all p-values are listed in Appendix B, tables B.2-B.4):

Erythrocytes and precursor cells (%): Erythrocytes were oval cells with an eosinophilic cytoplasm and elongated and centrally located nucleus (Figure 3.3A). Erythroid precursors were larger than the mature cells and had a larger nucleus: cytoplasmic ratio when compared to the more mature cells. There was no clear pattern of change in percentage of these cells for the different fish groups during the trial. However, comparing the smaller fish at 11°C with the larger fish at 20°C, they were significantly higher in the latter group at 14 weeks post temperature change (p-value=0.017).

Thrombocytes (%): Thrombocytes had a spindle, pyriform, or oval shape and a condensed nucleus that followed the cytoplasmic shape (Figure 3.3B). There was a significantly higher thrombocyte percentage in the smaller fish kept at the higher temperature at times 2 and 3 (6 and 14 weeks post temperature change) compared to the corresponding fish at 11°C. For all groups except for the smaller fish at 20°C, the percentage of thrombocytes was significantly lower at the end of the trial than at time 1 (p-value<0.005).

Lymphocytes (%): These cells had a round shape, with a large nucleus to cytoplasm ratio and a strongly basophilic cytoplasm (Figure 3.3C). In general, both sizes of fish housed at 20°C showed higher lymphocyte percentages than the corresponding fish housed at 11°C (Figure 3.4A); this difference was statistically significant for both sizes at 3 weeks post temperature change. There was a significantly different mean

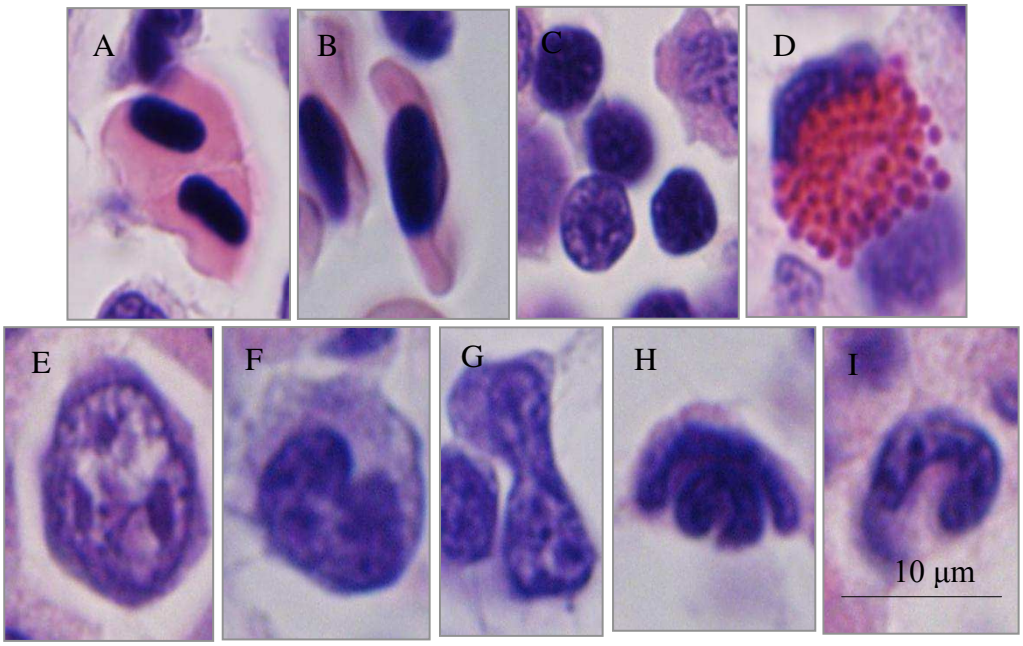

Figure 3.3. Juvenile A. brevirostrum meningeal myeloid tissue cells, Hematoxylin & Eosin. A) erythrocytes, B) thrombocytes, C) lymphocytes, D) granular eosinophilic cell, E) blast cell, F) and G) myeloid progenitor cells, H) immature heterophil and I) mature heterophil.

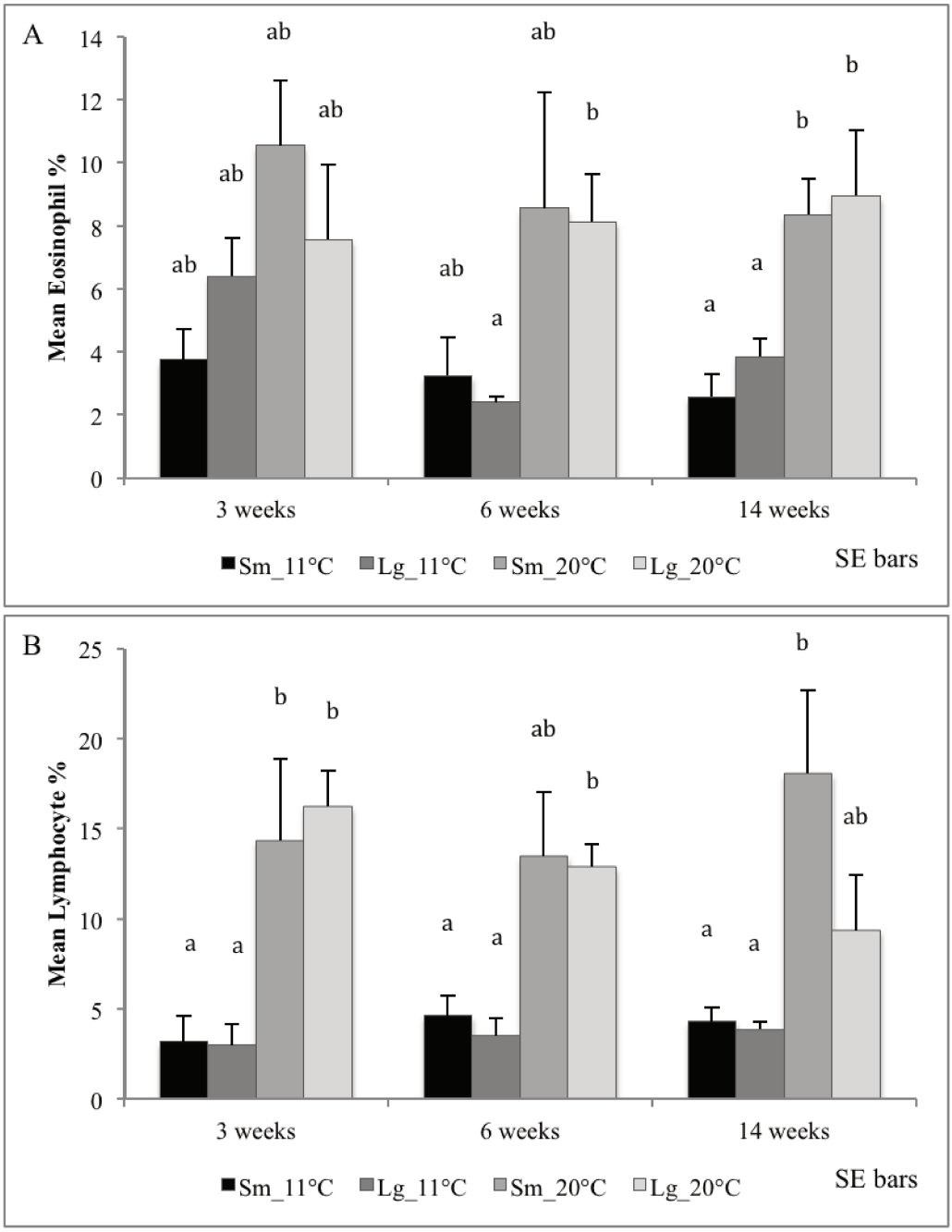

Figure 3.4. Meningeal myeloid tissue (A) eosinophil % and (B) lymphocyte % for the different groups of A. brevirostrum (smaller and larger sizes held at 11°C and 20°C). n=6; different letters signify statistically significant differences between groups.

There lymphocyte percentage between smaller fish at 11°C and larger fish at 20°C (p-value=0.001). There were no significant differences between both sizes of fish kept at the same temperatures and no significant differences for the different groups over time.

Eosinophils (%): These cells had a cytoplasm packed with brightly stained oval eosinophilic granules that partly obscured the nucleus that was located in an eccentric position of the cell. These cells were easily identified in the meningeal myeloid tissue (Figures 3.1B and 3.3D) and, in general, both sizes of fish had a higher percentage of eosinophils at 20°C than they did at 11°C (Figure 3.4B). This percentage was significantly greater in the larger fish at 6 weeks post temperature change (p-value=0.047) and in the smaller fish after 14 weeks (p-value=0.012), when compared to the corresponding groups at 11°C. Comparing smaller fish at 11°C with larger fish at 20°C, the eosinophil percentage was significantly higher in the latter group at 14 weeks post temperature change (p-value=0.014). There were no significant differences between both sizes of fish kept at the same temperatures or over time.

Blast cells (%): These cells were round in shape, with a large nucleus and prominent nucleoli and were generally larger than the remaining cells (Figure 3.3E). The smaller fish kept at 11°C had a significantly higher percentage of blast cells when compared to all of the other groups combined (p-value=0.03).

Myeloid progenitor cells (%): These cells had prominent nucleoli and an irregularly shaped nucleus, with intermediate characteristics between blast cells and immature heterophils (Figure 3.3F and 3.3G). The percentage of myeloid cells was higher for fish kept at 11°C for both sizes when compared to the corresponding size at 20°C (Figure 3.5A). This difference was statistically significant at 3 and 14 weeks post temperature change for both sizes of fish. The same occurred when comparing the smaller fish at

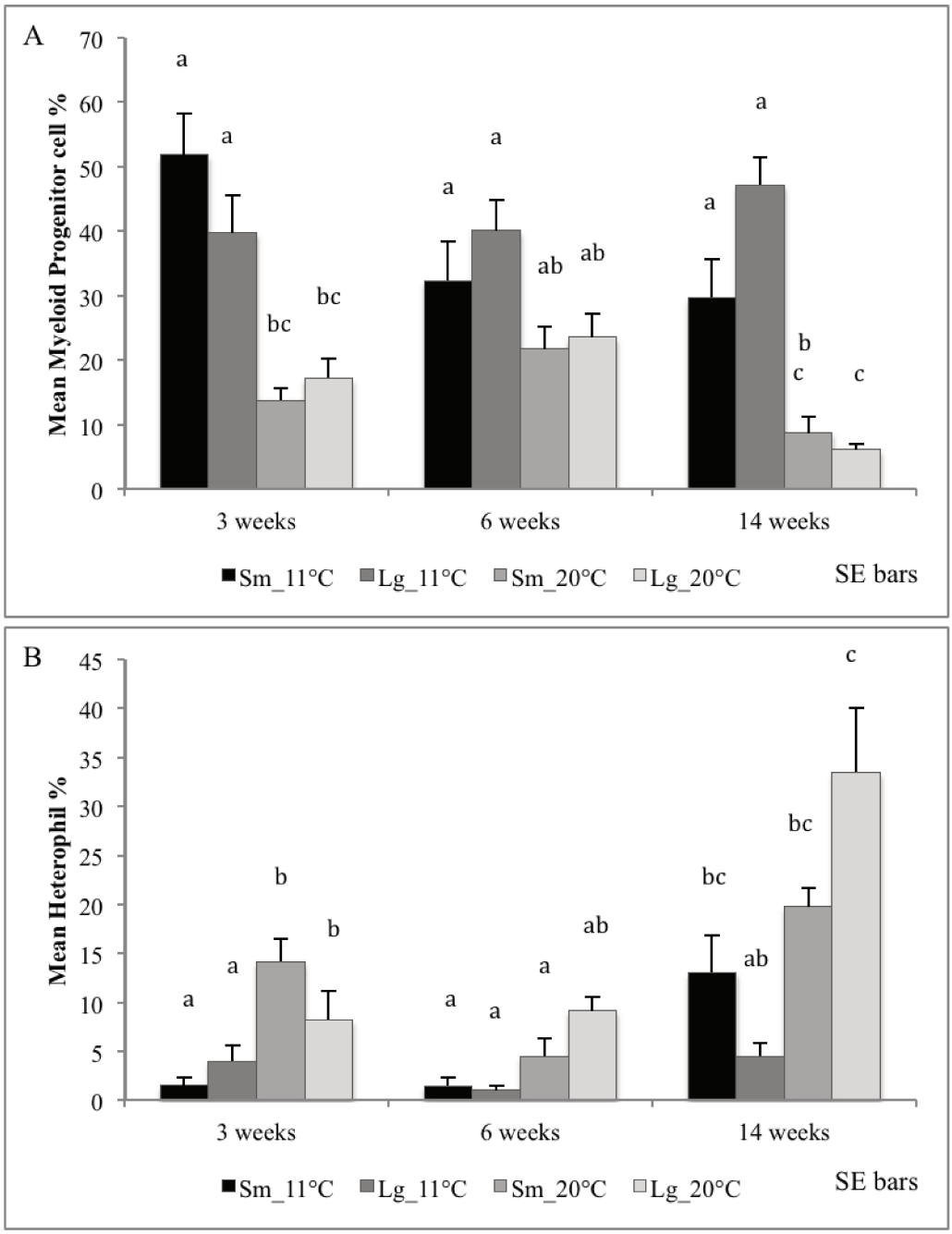

Figure 3.5. Meningeal myeloid tissue (A) myeloid progenitor cell % and (B) heterophil % for the different groups of A. brevirostrum (smaller and larger sizes held at 11°C and 20°C). n=6; different letters signify statistically significant differences between groups.

11°C with larger fish at 20°C (p-values<0.005). There were, however, no significant differences over time for the different groups except for the larger fish at 20°C between 6 and 14 weeks, where it was lower in the fish in the latter time point (p-value=0.003). There were no significant differences between both sizes of fish kept at the same temperatures.

Immature/band heterophils (%): These cells had a horseshoe-shaped nucleus with parallel sides and no constrictions in the nuclear membrane (Figure 3.3H). The only significant difference in the percentage of this cell type occurred between smaller fish at 11°C and large fish at 20°C at 14 weeks (p-value=0.015), where it was significantly higher in the smaller fish. There were no significant differences between both sizes of fish kept at the same temperatures and no change seen in these cells over time for the different groups.

Heterophils (%): These cells had a horseshoe-shaped nucleus with variable degrees of indentation and constriction around its perimeters, assuming different shapes (Figure 3.3I). The nuclear shape varied from reniform to fully segmented. The heterophil percentage was generally higher in both sizes of fish kept at 20°C and significantly higher in the smaller fish at 3 weeks and in the larger fish at 6 and 14 weeks (Figure 3.5B). There were no significant differences between both sizes of fish kept at the same temperatures. For heterophils, there was a significant difference in percentage between smaller fish at 11°C and larger fish kept at 20°C at 6 weeks post temperature change (p-value<0.005).

3.4.1.2. Spleen

All p-values for this analysis are listed in Appendix B, tables B.2-B.4. The morphometric analysis of the spleen showed a significantly greater white pulp percentage in the smaller fish kept at 20°C than in the fish housed at 11°C at 3 weeks post temperature change (p-value<0.005) (Figure 3.6). Also at this time, the smaller fish at 11°C had a significantly lower white pulp percentage when compared with the larger fish at 20°C (p-value=0.015). However, these differences disappeared over time and by the end of the trial all groups had a similar white pulp percentage.

3.4.1.3. Thymus

The thymus had a cortical area and a visible less densely packed medulla (Figure 3.1). Based on the serial sections of the small fish kept at 11°C, the within-fish proportion of variation was 57.5% and the between-fish proportion of variation was 42.5%. All p-values for this analysis are listed in Appendix B, tables B.2-B.4. There were no significant differences between the thymus areas between the different groups or over time (p-values are 0.08 and 0.34, respectively) without adjusting for a reference organ. The Pearson correlations between the average thymus area and the notochord and the average thymus area and the brain were weak (0.392 and -0.424, respectively). The notochord displayed more regular structure and was easier to accurately measure in terms of area and was, as such, chosen as the reference organ. There was a weak significant difference in the notochord area over time (p-value=0.047); however, when adjusting for the notochord, there were still no significant differences in the thymus areas between groups or over time (p-values are 0.38 and 0.84, respectively).

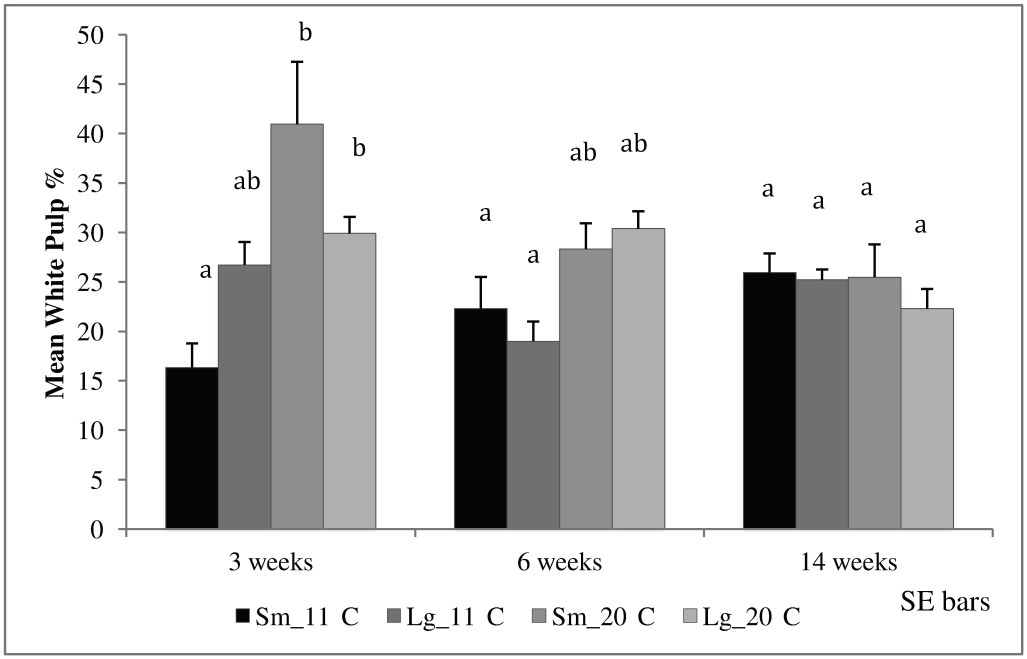

Figure 3.6. Splenic white pulp % for the different groups of A. brevirostrum (smaller and larger sizes held at 11°C and 20°C). n=6; different letters signify statistically significant differences between groups.

3.4.2. Gene expression

3.4.2.1. Spleen

IRF-1 and IRF-2: Both genes showed a similar relative expression profile (Figures 3.7A and 3.7B, respectively). Overall, their expression was significantly different between the groups (p-value =0.007), being higher in the fish kept at 20°C. However, these differences disappeared over time for both genes and by the end of the trial all groups had a similar gene expression.

MMP-9: No significant differences were seen for this gene for the different groups throughout the trial (Appendix C - Figure C.2A).

3.4.2.2. Skin

IRF-1 and IRF-2: Both genes showed a similar profile, with a significantly higher expression in the fish kept at 11°C at 3 and 6 weeks post temperature change (Figures 3.8A and 3.8B, respectively). However, these differences also disappeared over time for both genes and by the end of the trial all groups had similar IRF-1 and IRF-2 expression.

MMP-9: No significant differences were seen for this gene in either tissue for the different groups throughout the trial (Appendix C - Figure C.2B).

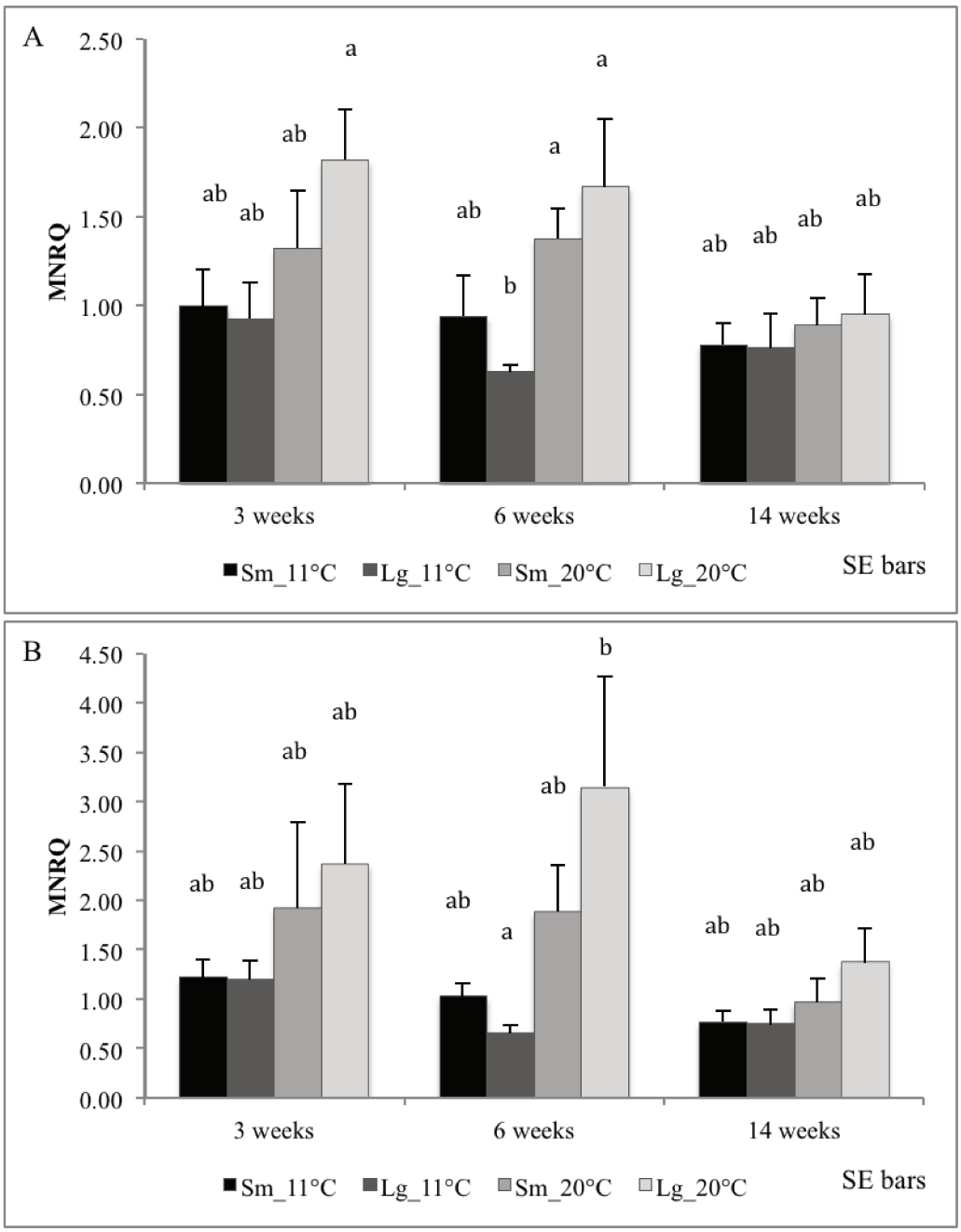

Figure 3.7. IRF-1 and IRF-2 gene expression in the spleen (A and B) for the different groups of A. brevirostrum (smaller and larger sizes held at 11°C and 20°C). n=6; different letters signify statistically significant differences between groups. MNRQ (Mean Normalized Relative Quantity).

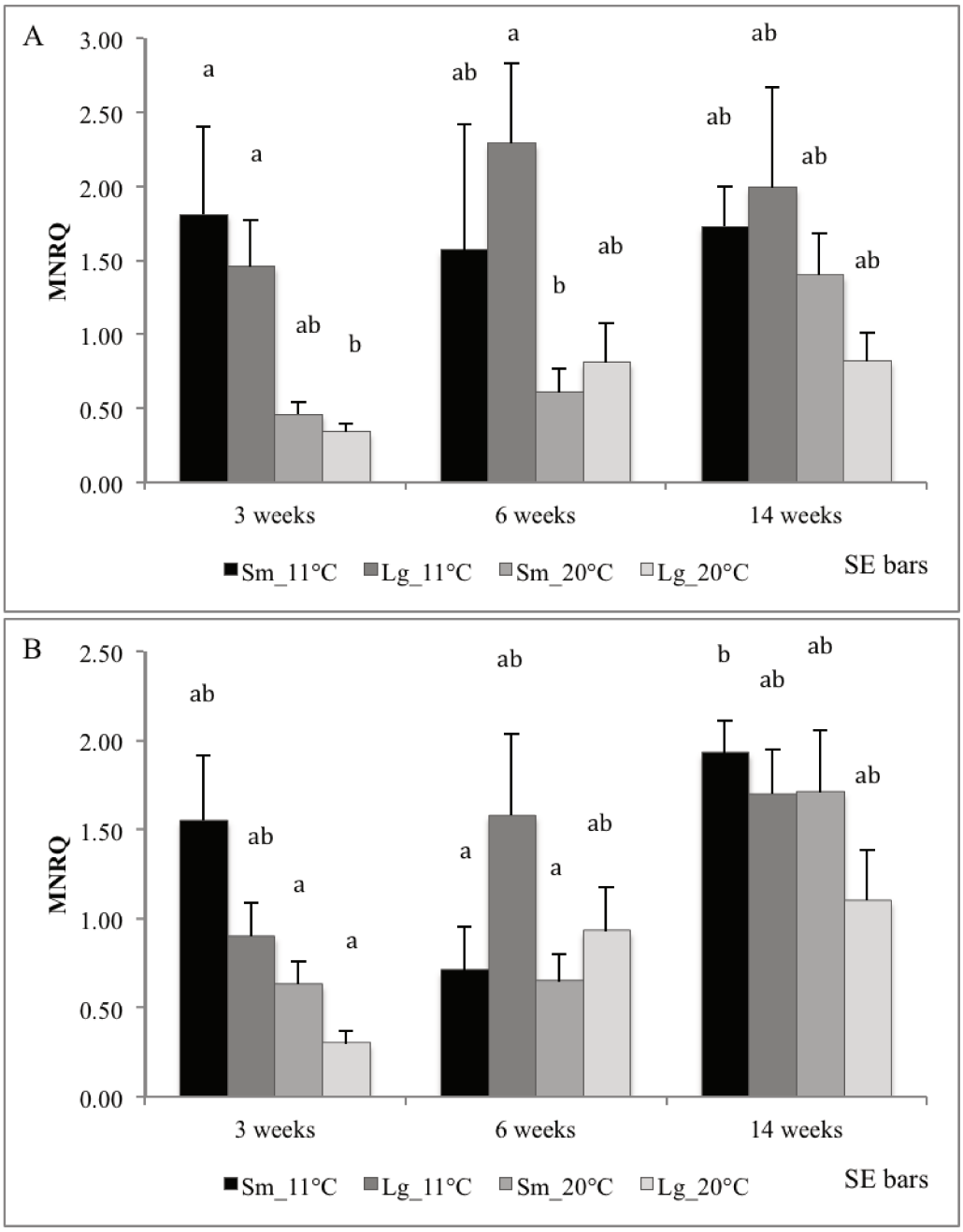

Figure 3.8. IRF-1 and IRF-2 gene expression in the skin (A and B) for the different groups of A. brevirostrum (smaller and larger sizes held at 11°C and 20°C). n=6; different letters signify statistically significant differences between groups. MNRQ (Mean Normalized Relative Quantity).

3.4.3. Parasite exposure

Adult female D. oblongum were observed to be taken up through the mouth of sturgeon. Since the parasite is often found in the oral cavity of the host and no previous research has been done regarding route of infection, it was hypothesized that it may be oral. However, careful physical examination of each fish after exposure to D. oblongum adults and copepodids revealed no gross evidence of parasite attachment on any of the fish. Packed cell volumes (PCVs) were tested before and after the parasite exposure as a further measure of potential effects the exposure may have had on the fish. Aside from no viable infection, PCVs were not significantly different between groups (Appendix C - Figure C.1).

3.5. Discussion

At the beginning of the trial, both sizes of fish had a significantly different weight (size) and the smaller fish kept at 20°C showed the highest growth of all groups over time (p-value<0.005). Considering size strongly influences immune system development[12], a more developed immunological response was expected in the larger fish in this study. The morphology of shortnose sturgeon blood cells was similar to that of other fishes and described based on prior sturgeon references[26-32]. As stated, the parasite exposure was unsuccessful and since the PCVs prior to and after the parasite exposure showed that the values for most fish were within the published reference interval[29], that there were no significant differences between exposed and unexposed groups, and both groups experienced normal feeding and swimming behavior, it is likely that the exposure had no effect on the final sampled tissues in this study.

3.5.1. Light microscopy

3.5.1.1. Meningeal myeloid tissue

The meningeal tissue is a unique hemopoietic site in sturgeon located over the medulla oblongata and it occupies the entire space between the brain and cartilaginous skull capsule in a saddle-like manner[33,23]. It is mainly a granulopoietic tissue with erythroid and lymphoid cell lines in different stages of maturation[23]. Histologically, the structure of the meningeal tissue resembles the hemopoietic bone marrow in mammals[23]. Studying how temperature and size influenced cell percentages in this tissue was relevant to help determine the optimal immune development in sturgeon of this age.

Erythrocyte and precursor cells (%): Even though there was no clear pattern in erythrocyte and erythrocyte precursor cell percentages in the different groups, the larger cell percentage in the larger fish was likely related to both fish size and the higher water temperature. Both higher vascularization of the meningeal tissue and production of red blood cells may occur in the larger fish but such analysis extends beyond the scope of this study.

Thrombocytes (%): For the smaller fish, thrombocyte percentage in the meningeal tissue was significantly higher at 20°C than at 11°C at 6 and 14 weeks post temperature change. Research done on rainbow trout has shown that water temperature strongly influences blood thrombocyte percentages[34]. Likewise, water temperature influenced thrombocyte percentages in sturgeon meningeal tissue. Stress increases the percentage of circulating thrombocytes in rainbow trout[35]. The significant decrease in the meningeal thrombocyte percentages for all groups by the end of the trial (except for the smaller fish at 20°C, where the decrease was non-significant) suggests that sturgeon may have produced more thrombocytes after a stress event such as transportation or establishing

tank hierarchies from the hatchery to our facility, and eventually acclimated over time. However, there was no concurrent analysis of thrombocytes in the other hemopoietic organs or in the blood in this study to confirm this.

Eosinophils and lymphocytes (%): In shortnose sturgeon blood, lymphocytes are classified according to their size as small or large[29]. Considering that lymphoid cell progenitors decrease in size and cytoplasmic basophilia as they mature and increase condensation of nuclear chromatin[32], lymphocytes of both sizes in several stages of development present in the meningeal myeloid tissue were grouped for counting purposes. In general, both sizes of fish had a higher percentage of lymphocytes at 20°C than at 11°C, indicating that temperature strongly influenced their production in the meningeal tissue. Based on the findings from Chapter 2 for the meningeal myeloid tissue, Atlantic sturgeon juveniles until 2895 dd showed similar lymphocyte percentages to those present in shortnose sturgeon kept at 11°C. However, these percentages increased 3-4 fold in the shortnose sturgeon groups kept at 20°C, suggesting temperature is much more important than age in stimulating lymphocyte development in sturgeon meningeal myeloid tissue. The same occurred for the eosinophils, supporting existing literature that fish kept at lower temperatures have a significantly lower number of lymphocytes when compared to similar sized fish at higher temperatures[10].

Blast cells (%): These are undifferentiated, mononuclear immature blood cells[26]. The smaller fish kept at 11°C showed a significantly higher blast cell percentage when compared to all the other groups combined. These are undifferentiated cells, and a higher percentage would be expected in the less developed fish, based on literature[10]. The results indicate that both size and temperature influenced immune cell development in the meningeal myeloid tissue in these fish.

Myeloid progenitor cells, immature and mature heterophils (%): Myeloid progenitor cells are precursors of neutrophils and monocytes and include several stages of development (promyelocytes, myelocytes and metamyelocytes) before they differentiate into monocytes or neutrophils (heterophils in sturgeon). Granulocytes decrease cellular size and cytoplasmic to nuclear ratio as they mature. Secondary specific granules appear in the myelocyte stage and allow differentiation of different granulocytic lineages. Metamyelocytes have elongated and bean-shaped nucleus before nuclear segmentation. For consistency, all of these intermediate cell types were grouped into this category[32]. The percentage of myeloid progenitor cells was higher for fish kept at 11°C for both sizes when compared to the corresponding size at 20°C, indicating that temperature strongly influenced the production of these cells. It is important to emphasize that if the increase in cell percentages found in the meningeal myeloid tissue in the fish kept at 20°C was a result of an immune response to a pathogen in the surrounding water, the myeloid progenitor cell percentage should have been increased in these fish when compared to the corresponding fish at 11°C, which was not the case. Both immature and mature heterophil percentages suggest that temperature strongly influenced their production and that fish kept at 20°C may have a faster heterophil maturation rate than the fish kept at 11°C. Accordingly, a higher percentage of mature cells was found in the larger fish kept at 20°C. Considering that the production and maturation of these cells is a continuous process, the results are consistent with the literature on teleosts[10], where the smaller animals kept at the lower temperature showed a higher proportion of less developed cell stages and less mature cells compared to the larger and more developed fish.

3.5.1.2. Spleen

In sturgeon, the spleen serves as a blood reservoir and it has immune functions, trapping circulating antigens and being involved in lymphopoiesis[23,24]. There was a significant difference between the smaller fish at both temperatures after 3 weeks, which suggests that rearing the smaller fish at a higher temperature might increase splenic white pulp percentage. However, by the end of the trial, all groups showed a similar white pulp percentage, which indicated that the effect of temperature was only transient and may have required from 6-14 weeks for the fish to acclimate to this temperature.

3.5.1.3. Thymus

Similar to what has been described in the literature[23,24], the thymus is located adjacent to the gill openings and it is lobulated. An outer cortex and an inner densely packed medulla were visible and the thymic parenchyma contained lymphocytes and reticular cells. The similarity between the morphological structure of the sturgeon thymus and that of higher vertebrates suggests that they are functionally similar[24], The aim of the morphometric analysis of the histological sections of the thymus was to morphologically compare lymphocyte production in response to temperature over time for both sizes of fish. In order to compare the different groups, it was necessary to assess what the variation in the thymus area was for individual fish within a group. The results showed that for the group selected (smaller fish at 11°C), the variation between the serial sections for the individual fish (within-fish variation) was greater than the variation between the different fish (between fish variation) of that group. This can be due to individual variability, to individual thymus involution rate (unknown in this species), to the use of 2-dimensional images of sections to assess a 3-dimensional organ and to the

averaged area of both left and right components of the thymus for each fish. Also, it is not known how the within-fish proportion of variation changes throughout the trial for the smaller fish at 11°C or for the remaining fish groups. Nonetheless, we attempted to compare the different groups by standardizing each fish with a reference organ present in the same section. However, using the notochord as a reference, there was still no difference between the fish groups. This may be due to a number of factors: there might truly be no difference in thymus size between the groups, the high within-fish proportion of variation in thymus size in each group and the weak correlation between the notochord (or brain) and the thymus might compromise the analysis and findings. Also, the involution rate of the thymus (unknown in sturgeon) might vary between fish and be influenced by temperature as well as fish size. More research is needed to elucidate the effect temperature and fish size have on lymphocyte production in the sturgeon thymus.

3.5.2. Gene expression

All p-values for this analysis are listed in Appendix B, tables B.2-B.4:

IRF-1 and IRF-2: IRF-1 and IRF-2 were recently characterized in paddlefish (PsIRF-1 and PsIRF-2) and their putative protein sequences are very similar to those of teleosts and mammals[36]. IRF-1 and IRF-2 are transcriptional factors involved in type I interferon (IFN) regulation. IRF-1 functions as an activator and IRF-2 suppresses IRF-1 function[37]. In healthy paddlefish (Polyodon spathula), these genes are expressed constitutively in several organs and are highly expressed in the spleen[36]. In the spleen of sturgeon kept at 20°C, the temperature-induced increase in the expression levels for both genes was similar to the morphometric white pulp percentage findings, suggesting that this organ responds differently to an increase in temperature. According to a study done in Atlantic

cod, temperature increase appeared to accelerate the spleen immune transcriptome in response to a viral mimic antigen, with IRF-1 and IRF-2 being up-regulated[38]. A similar effect may occur in sturgeon spleen but whether this enhancement would be a transient one would require further investigation.

The skin is an important organ in terms of iridoviral infections common in sturgeon[16]. In healthy Atlantic cod, IRF-1 was found to be significantly higher in the ventral region of the skin than in the dorsal region; this indicates that a basal expression level of these genes exists in uninfected teleost skin[39]. A study done in carp (Cyprinus carpio) showed that several immune genes including IFN-like genes were present in the skin and differential expression of these genes occurred in response to the protozoan ectoparasite, Ichthyophthirius multifiliis[40]. In this current study, IRF-1 and 2 appeared to have different expression profiles in the spleen and skin (higher at 20°C and higher at 11°C, respectively). This may be due to a lower expression of these genes in the skin following transfer to higher water temperatures (under 6 weeks) and, like the spleen, it is a transient effect. Higher percentages of white pulp in spleen and lymphocytes in the meningeal myeloid tissue at the higher temperature suggest that this may be in part due to, in the spleen at least, a positive interaction with IRF-1. Higher IRF-expressing cells in the skin at the lower temperature may be due to a reduced extravasation into skin of these same cell types and higher numbers maintained in the hemopoietic tissue (spleen and meninges) at the increased temperature. However, further research is needed.

MMP-9: It is involved in many physiological functions such as growth, development and wound healing[41]. In teleost fish, MMP-9 may be considered an active participant in the innate immune response[42]. MMP-9 is important in vascularization and remodeling of the extracellular matrix[43]. MMP-9 also plays a role during the initial phase and during

the later process of termination of the inflammatory response in carp[42]. Studies on Atlantic salmon responses to infection with sea lice (Lepeophtheirus salmonis) have shown significant changes of MMPs with infection level. MMP-9 specifically showed a delayed increase in the head kidney and spleen first noticed at 22 days post infection, but following immunostimulation there was an increased expression earlier on, associated with partial rejection of the parasite[19,44]. The lack of changes in MMP-9 expression in the skin in the current study was not surprising since the parasite infection was likely unsuccessful. D. oblongum is a marine parasitic copepod known to attach to the gills (operculum) and pectoral, dorsal, and anal fins of infested fish. Considering that the infection mechanism is unknown for this parasite, it was interesting to observe that one of the paths by which they are taken up by the host is via their oral cavity. D. oblongum is a common parasite of wild sturgeon and can have a serious impact on their hosts due to stress or water loss through infestation, which compromises the epithelial barrier[45]. Although the gills were a possible route of infection, the exposure was most likely unsuccessful, with no effects observed on the host. It might be that this parasite is only infective in saltwater and therefore developing better challenge models would be a useful endeavor. Since tolerance to salinity in sturgeon increases with size and even 16 month-old shortnose sturgeon are not fully adapted to saltwater[46], using 3 to 4 year old Atlantic and shortnose sturgeon with higher salinity tolerance as parasite hosts may be reasonable[47]. Also, a histological analysis of the skin lesions caused by the parasites could be done. There were no other factors within the study that should have stimulated a wounding/inflammatory response in these fish. Furthermore, although wound healing is affected by temperature in teleosts[48], the temperature differences observed here did

not appear to have any effect on splenic or skin MMP-9 expression in shortnose sturgeon.

3.6. Conclusions

This study showed that temperature strongly influenced immune organ composition in juvenile sturgeon, regardless of size. The meningeal myeloid tissue appeared to be the most responsive, with the more specific later cell stages being significantly higher at the higher temperature. Although lymphocyte percentages were lower at 11°C, there was not enough evidence to confirm our hypothesis that sturgeon kept at lower temperatures relied more heavily on innate immune responses. The effect on the spleen was also present, although it was transient. The gene expression data for IRF-1 and IRF-2 genes showed positive associations with the splenic white pulp percentage at the higher water temperature and will require further investigation. Overall, the findings in this study seem to support a stronger immunological capacity in the fish kept at 20°C, regardless of the fish sizes considered here. Also, smaller fish kept at this temperature were able to grow faster than the other groups and compensate for prior low growth rates. These findings are useful for sturgeon hatcheries and farms, where an increase in the temperature at which the fish are reared increases growth rates and may help increase immunological development and decrease early life stage mortality in these species.

3.7. References

1. Bronzi, P., Rosenthal, H., & Gessner, J. 2011. Global sturgeon aquaculture production: An overview. J. Appl. Ichthyol. 27, 169-175.

2. Kynard, B. 1997. Life history, latitudinal patterns, and status of the shortnose sturgeon, Acipenser brevirostrum. Environ. Biol. Fishes. 48, 319-334.

3. Kieffer, M.C. & Kynard, B. 1993. Annual movements of shortnose and Atlantic sturgeons in the Merrimack river, Massachussets. Trans. Am. Fish. Soc. 122, 1088-1103.

4. Piper, R.G., McElwain, I.B., Orme, L.E., McCraren, J.P., Fowler, L.G. & Leonard, J.R. 1982. Fish Hatchery Management. Department of the Interior, U.S. Fish and Wildlife Service. Washington, D. C. USA. Pp. 3-4.

5. Cushing, J.E. Jr. 1942. An effect of temperature upon antibody-production in fish. J. Immunol. 45, 123-126.

6. Hardie, L.J., Fletcher, T.C. & Secombes, C.J. 1994. Effect of temperature on macrophage activation and the production of macrophage activating factor by rainbow trout (Oncorhynchus mykiss) leucocytes. Dev. Comp. Immunol. 18, 57-66.

7. Alcorn, S.W., Murray, A.L. & Pascho, R.J. 2002. Effects of rearing temperature on immune functions in sockeye salmon (Oncorhynchus nerka). Fish Shellfish Immunol. 12, 303-334.

8. Rijkers, G.T., Frederix-Wolters, E.M.H. & Van Muiswinkel, W.B. 1980. The immune system of cyprinid fish. Kinetics and temperature-dependence of antibody-producing cells in carp (Cyprinus carpio). Immunology. 41, 91-97.

9. Carlson, R.E., Baker, E.P. & Fuller, R.E. 1995. Immunological assessment of hybrid striped bass at three culture temperatures. Fish Shellfish Immunol. 5, 359-373.

10. Stolen, J.S., Draxler, S. & Nagle, J.J. 1984. A comparison of temperature-mediated Immunomodulation between two species of flounder. Immunol. Invest. 13, 245-253.

11. Dadswell, M.J. 1979. Biology and population characteristics of the shortnose sturgeon, Acipenser brevirostrum LeSueur 1818 (Osteichthyes: Acipenseridae), in the Saint John River estuary, New Brunswick, Canada. Can. J. Zool. 57, 2186-2210.

12. Johnson, K.A., Flynn, J.K. & Amend, D.F. 1982. Onset of immunity in salmonid fry vaccinated by direct immersion in Vibrio anguillarum and Yersinia ruckeri bacterins. J. Fish Dis. 5, 197-205.

13. Hedrick, R.P., McDowell, T.S., Groff, J.M., Yun, S. & Wingfield, W.H. 1991. Isolation of an epitheliotropic herpesvirus from white sturgeon Acipenser transmontanus. Dis. Aquat. Organ. 11, 49-56.

14. Georgiadis, M.P., Hedrick, R.P., Johnson, W.O., Yun, S. & Gardner, I.A. 2000. Risk factors for outbreaks of disease attributable to white sturgeon iridovirus and white sturgeon herpesvirus-2 at a commercial sturgeon farm. Am. J. Vet. Res. 61, 1232-1240.

15. Hua, Y. & Wang, D. 2005. A review of sturgeon virosis. J. Forestry Res. 16, 79-82.

16. Kurobe, T., MacConnell, E., Hudson, C., McDowell, T.S., Mardones, F.O. & Hedrick, R.P. 2011. Iridovirus Infections among Missouri River Sturgeon: Initial characterization, transmission, and evidence for establishment of a carrier state. J. Aquat. Anim. Health. 23, 9-18.

17. Drennan, J.D., LaPatra, S.E., Swan, C.M., Ireland, S. & Cain, K.D. 2007. Characterization of serum and mucosal antibody responses in white sturgeon (Acipenser transmontanus Richardson) following immunization with WSIV and a protein hapten antigen. Fish Shellfish Immunol. 23, 657-669.

18. Geist, D.R. & Brown, R.S. 2005. Movement, swimming speed, and oxygen consumption of juvenile white sturgeon in response to changing flow, water temperature, and light level in the Snake River, Idaho. Trans. Am. Fish. Soc. 134, 803-816.

19. Covello, J.M., Friend, S.E., Purcell, S.L., Burka, J.F., Markham, R.J.F., Donkin, A.W., Groman, D.B. & Fast, M.D. 2012. Effects of orally administered immunostimulants on inflammatory gene expression and sea lice (Lepeophtheirus salmonis) burdens on Atlantic salmon (Salmo salar). Aquaculture. 366–367, 9-16.

20. Bancroft, J.D. & Cook, H.C. 1995. Manual of Histological Techniques and their diagnostic application. Churchill Livingstone, Singapore. Pp. 27.

21. Chomczynski, P. & Saccho, N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162, 156–159.

22. Livak, K.J. & Schmittgen, T.D. 2001. Analysis of relative gene expression data using real- time quantitative PCR and the $2^{-\Delta\Delta Ct}$ Method. Methods. 25, 402-408.

23. Fänge, R. 1986. Lymphoid organs in sturgeons (Acipenseridae). Vet. Immunol. Immunopathol. 12, 153-161.

24. Lange, M.A., Govyadinova, A.A. & Khrushchev, N.G. 2000. Study on localization of hemopoietic tissue in sturgeon. Rus. J. Dev. Biol. 31, 372-376.

25. Palić, D., Beck, L., Palić, J. & Andreasen, C. 2011. Use of rapid cytochemical staining to characterize fish blood granulocytes in species of special concern and determine potential for function testing. Fish Shellfish Immunol. 30, 646-652.

26. Dexter, T.M., Allen, T.D. & Lajtha, L.G. 1977. Conditions controlling the proliferation of haemopoietic stem cells in vitro. J. Cell Physiol. 91, 335-344.

27. Kawamoto, H. & Minato, N. 2004. Cells in focus: Myeloid cells. Int. J. Biochem. Cell Biol. 36, 1374-1379.

28. Thrall, M.A. 2004. Veterinary Hematology and Clinical Chemistry. Lippincott Williams and Wilkins. Printed in the US. Pp. 125-134.

29. Knowles, S., Hrubec, T.C., Smith, S.A. & Bakal, R.S. 2006. Hematology and plasma chemistry reference intervals for cultured shortnose sturgeon (Acipenser brevirostrum). Vet. Clin. Pathol. 35, 434.

30. Eurell, J.A. & Frappier, B.L. 2006. Dellman's Textbook of Veterinary Histology. 6th edition, Blackwell Publishing, Iowa, USA. Pp. 61-66, 70-75, 136,137, 140, 141, 147-152.

31. Samuelson, D.A. 2007. Textbook of Veterinary Histology. Saunders Elsevier. China. Pp. 130-158.

32. Weiss, D.J. & Wardrop, K.J. 2010. Schalm's Veterinary Hematology. 6th edition. Wiley-Blackwell Publishing, Iowa, USA. Pp. 996-1002.

33. Scharrer, E. 1944. The histology of the meningeal myeloid tissue in the Ganoids Amia and Lepisosteus. Anat. Rec. 88, 291-310.

34. Kfoury-Junior, J.R., Akutsu, T. & Okamoto, N. 2006. Influence of rearing temperature changes on thrombocytes aggregation in rainbow trout. Braz. J. Vet. Res. Anim. Sci. 43, Suppl., 23-27.

35. Casillas, E. & Smith, L.S. 1977. Effect of stress on blood coagulation and haematology in rainbow trout (Salmo gairdneri). J. Fish Biol. 10, 481-491.

36. Xiaoni, G., Zhuo, C., Xuzhen, W., Dengqiang, W. & Xinwen, C. 2012. Molecular cloning and characterization of interferon regulatory factor 1 (IRF-1), IRF-2 and IRF-5 in the chondrostean paddlefish Polyodon spathula and their phylogenetic importance in the Osteichthyes. Dev. Comp. Immunol. 36, 74-84.

37. Harada, H., Fujita, T., Miyamoto, M., Kimura, Y., Maruyama, M., Furia, A., Miyata, T. & Taniguchi, T. 1989. Structurally similar but functionally distinct factors, IRF-1 and IRF-2, bind to the same regulatory elements of IFN and IFN-inducible genes. Cell. 58, 729-739.

38. Hori, T.S., Gamperl, A.K., Booman, M., Nash, G.W. & Rise, M.L. 2012. A moderate increase in ambient temperature modulates the Atlantic cod (Gadus morhua) spleen transcriptome response to intraperitoneal viral mimic injection. BMC Genomics. 13, 431.

39. Caipang, C.M.A., Lazado, C.C., Brinchmann, M.F., Rombout, J.H.W.M. & Kiron, V. 2011. Differential expression of immune and stress genes in the skin of Atlantic cod (Gadus morhua). Comp. Biochem. Physiol. Part D Genomics Proteomics. 6, 158-162.

40. Gonzalez, S.F., Chatziandreou, N., Nielsen, M.N., Li, W., Rogers, J., Taylor, R., Santos, Y. & Cossins, A. 2007. Cutaneous immune responses in the common carp detected using transcript analysis. Mol. Immunol. 44, 1664-1679.

41. Van den Steen, P.E., Dubois, B., Nelissen, I., Rudd, P.M., Dwek, R.A. & Opdenakker, G. 2002. Biochemistry and molecular biology of gelatinase B or matrix metalloproteinase-9 (MMP-9). Crit. Rev. Biochem. Mol. Biol. 37, 375-536.

42. Chadzinska, M., Baginski, P., Kolaczkowska, E., Savelkoul, H. F. J. & Verburg-van Kemenade, B.M.V. 2008. Expression profiles of matrix metalloproteinase 9 in teleost fish provide evidence for its active role in initiation and resolution of inflammation. Immunol. 125, 601-610.

43. Soler, R.C., Gui, Y., Linask, K.K. & Muschel, R.J. 1995. MMP-9 (gelatinase B) mRNA is expressed during mouse neurogenesis and may be associated with vascularization. Brain Res. Dev. Brain Res. 88, 37-52.

44. Skugor, S., Glover, K.A., Nilsen, F. & Krasnov, A. 2008. Local and systemic gene expression responses of Atlantic salmon (Salmo salar L.) to infection with the salmon louse (Lepeophtheirus salmonis). BMC Genomics. 9, 498-516.

45. Fast, M.D., Sokolowski, M.S., Dunton, K.J., & Bowser, P.R. 2009. Dichelesthium oblongum (Copepoda: Dichelesthiidae) infestation in wild-caught Atlantic sturgeon, Acipenser oxyrinchus oxyrinchus. ICES J. Mar. Sci. 66, 2141-2147.

46. Jarvis, P.L & Ballantyne, J.S. 2003. Metabolic responses to salinity acclimation in juvenile shortnose sturgeon Acipenser brevirostrum. Aquaculture. 219, 891-909.

47. Krayushkina, L.S. 2006. Considerations on evolutionary mechanisms of osmotic and ionic regulation in Acipenseridae: an overview. J. App. Ichthyol. 22 (Suppl. 1), 70-76.

48. Anderson, C.D. & Roberts, R.J. 1975. A comparison of the effects of temperature on wound healing in a tropical and a temperate teleost. J. Fish Biol. 7, 173-182.

Chapter 4.0 General Discussion

4.1. Discussion

Sturgeon are harvested worldwide for their meat and eggs (sold as caviar) and are among the most economically important fish species worldwide[1]. Considering the phylogenetically primitive status of Acipenserids, that all extant sturgeon species are currently listed under the CITES convention, and that how little is known about sturgeon immune responses to external stressors, it is important to study both innate and adaptive immune components in these species to improve husbandry practices and animal welfare in sturgeon aquaculture and in the wild.

One of the main challenges in sturgeon farming is the culture of larvae, considered to be one of the most critical and difficult stages in intensive sturgeon farming and often associated with a high mortality rate during and after yolk sac absorption and onset of feeding[2,3]. Efforts to minimize mortality rates during this phase are based on providing adequate nutrition to the larvae[3], but during this period the larvae must rely solely on innate mechanisms until they develop a functional adaptive immune system, which may take several weeks to months to develop[4]. To begin addressing the immune competence of larvae and juvenile sturgeon, it is important to determine when the immune organs first appear in these phylogenetically primitive fish and how they develop during these critical life stages. Relevant immune organs such as the meningeal myeloid tissue, the spleen and the thymus were characterized morphologically in Atlantic sturgeon during the first 5 months of life (until 2895 dd), since hatching. Each organ was characterized by light microscopy (LM) and transmission electron microscopy (TEM) and their cell populations described for all age groups collected. EM was the technique of choice for the initial morphological description and for the differential cell counts in the immune

organs studied in such young small fish. In salmon, the spleen and the thymus are present in larvae around the onset of feeding[5]. In sturgeon, the spleen was first visible approximately 400 dd after the onset of feeding (at 541 dd) and the meningeal myeloid tissue and the thymus at 768 dd (first appearing between 541 dd and 768 dd). The fact that lymphocytes were also not present in Atlantic sturgeon prior to 768 dd suggests that effective immune responses may be significantly delayed in sturgeon in comparison to teleosts such as salmonids.

All cell types considered for each of these organs were present in one or more of the age groups analyzed; however, lymphocyte populations did not change over time in the meningeal myeloid tissue in Atlantic sturgeon kept at 11°C. When looking at the cell percentages in shortnose sturgeon kept at 20°C, it would be interesting to see if lymphocyte numbers could be increased earlier in development if the fish were exposed to a higher temperature earlier on, possibly emphasizing the relevant role temperature increase would have on immunological development. This may be an interesting area for future research in juvenile sturgeon. For the meningeal myeloid tissue, heterophil and eosinophil percentages were greater in the larger fish (950 and 2895 dd samples) and the percentages of reticular cells and undifferentiated cells were significantly higher in the smallest animals, possibly reflecting a less developed immune system in these fish. The morphologic features of the spleen were similar to that previously described in sturgeon[7] and the most relevant changes during spleen development in Atlantic sturgeon were in the heterophil percentages, which were significantly higher in the oldest fish (2895 dd). There were also no significant changes in splenic mature lymphocyte percentages (absent in the youngest fish) but there was a significantly higher undifferentiated cell percentage in the younger fish compared to the oldest. Again, this emphasizes a less

developed spleen in the smaller animals and progressive cell differentiation and maturation over time in this tissue. The relatively high percentage of splenic undifferentiated cells in the 950 dd fish might indicate that they are less immunologically developed than the equivalently sized 2895 dd fish despite their size and that age might also play an important role in the immunity at such early life stages. In the thymus, the predominant cell type was the lymphocyte, found in different stages of maturation throughout the organ. The only significant difference found was in undifferentiated cell percentages (highest in the oldest group), which might reflect a more active proliferative thymus in the older animals. However, further research would be needed to elucidate this since there were no significant differences in the percentages of apoptotic or mitotic cells between the different groups (even though they were higher in the oldest age group). The thymus is the main lymphocyte producing organ in fish. When it was first visible in the samples studied (768 dd) and in the older age groups, its architecture was similar to that described in other fish[6]. However, unlike teleosts[5], it is not likely that an adaptive immune response occurs in sturgeon larvae at onset of feeding, since the thymus appears approximately 600 dd after that. To summarize the ontogeny study, clear ultrastructural organization of these immune organs was observed only in samples older than 541 dd (33 dph), which might suggest that an effective immune response might not occur in the early life stages prior and during the onset of feeding. However, further research is needed to assess the immune development of these and other immune organs (kidney and spiral valve) and overall immune competence in these life stages, which may be influenced by other factors such as the presence of maternal immune factors. Further research is also needed to assess immune development in Atlantic sturgeon juveniles and following pathogen challenge or immunostimulation.

Once the ontogeny of these immune organs was studied in sturgeon larvae and juvenile fish, it was then feasible to characterize them in older animals and assess their response under different environmental conditions. Since the factor that most influences development, growth and physiological functions in fish is water temperature, a temperature trial was done. The meningeal myeloid tissue, spleen, thymus and skin were studied in 2 different sizes of 8 to 9 month-old juvenile shortnose sturgeon, kept at either 11°C or 20°C. Given the lack of morphological studies of these organs under such conditions, paraffin-embedded specimens were stained with H&E and a differential cell count was done in meningeal myeloid tissue. This was possible due to the nature of this tissue, where the cells are spread out in animals of this age and individual cell identification was possible. Temperature strongly influenced immune cell percentages in the meningeal myeloid tissue (lymphocyte, eosinophil and heterophil percentages were significantly higher at the higher temperature), regardless of the fish sizes considered. Due to the cell density in the spleen, a morphometric analysis of splenic white pulp was performed, showing an initial increase at the higher temperature, but it was a transient response. A similar effect was seen for IRF-1 and IRF-2 expression in this organ and further research is required to better characterize the expression level of these genes in the spleen. As done for salmonids[8] it would be interesting to assess the nature and persistence of an antiviral responses in shortnose surgeon following injection of poly inosinic:cytidylic acid (poly I:C), kept under different water temperatures. No changes were seen in the expression of MMP-9 in the spleen.

The thymus was the most challenging organ to study morphologically for numerous reasons. The main goal of this characterization was to determine if such an analysis would allow for the indication of temperature-induced lymphocyte percentage

changes in this organ. Due to the high density of the cells in this organ and the fact that most cells present were lymphocytes and epithelial reticular cells, a differential cell count was not useful. A morphometric analysis would potentially allow for an assessment of the change in thymus size between the different groups. However, there were several limitations to our analysis. The first challenge consisted in measuring the volume of a bilateral organ based on 2-dimensional histology sections, in different-sized fish, with unknown individual thymic growth rates. The lack of a morphometric ratio (as done for the spleen white pulp area and total spleen area) implied that only absolute values could be compared for the different fish. Therefore, the area where the thymus section was cut had to be similar between fish. Since that was difficult to guarantee for all animals, it was thought that a reference organ present in that same cut cross-section could adjust for such differences; the areas of the notochord and the brain were then measured for all sections analyzed. Another important aspect to consider was that the individual variability in thymus size between different fish of the same group was unknown, as was the involution rate for the thymus in sturgeon at this age and at different temperatures. The within species variability was determined to be too high within one of the groups, making it difficult to critically compare thymus size across the different groups. A statistical analysis was done nonetheless, and as expected, there were no differences between the thymus size for the different groups. Still, considering all the limitations present in this assessment, this study was useful in addressing practical limitations that hinder the successful morphological analysis of the thymus and will be useful for future studies.

Regarding gene expression in the skin for the different groups, the goal was to study the effect a change in temperature would have on skin immunity and what effects

an ectoparasite would induce on the skin immune response. The anatomical location of the skin sample was based on the location where these parasites are often seen. Prior to the parasite exposure, IRF-1 was significantly higher in the fish kept at 11°C (over the first 6 weeks of the study), with IRF-2 expression generally having a similar profile to that of IRF-1 during the trial. After the parasite exposure, no differences were detected in the expression of MMP-9 between the groups but further research is required. A response at the gene level (together with macroscopic skin lesions) would have been suggestive of a successful parasite exposure. However, no parasites were detected in the skin or gills of the exposed fish at any time and the lack of change in MMP-9 gene expression in the skin was a further indication of an unsuccessful exposure. It is necessary to study the expression of this and other genes in different areas of sturgeon skin, as their baseline profile may change depending on where the skin samples are collected in the fish[19]. In the current study, the maintenance of adult female parasites and copepods in an experimental setting was attained, which had not been accomplished for this parasite in the past. Further exposure studies should be designed to better elucidate and characterize host immune responses to sturgeon parasites such as D. oblongum.

4.2. Conclusions

Due to the lack of knowledge regarding immune responsiveness in sturgeon, associated with their primitive phylogenetic status, the establishment of a timeline for the appearance and development of relevant immune organs was an essential step for further functional assessment of immune responses in these fish. The spleen was first visible at 541 dd and the meningeal myeloid tissue and thymus at 768 dd. All these organs were found to appear after the onset of feeding in sturgeon and less developed

cell stages were found in the younger animals. In older juvenile sturgeon, a higher temperature was shown to have a significant positive influence in the immune cell percentages in the meningeal myeloid tissue and in the spleen, even if it was a transient effect in the latter. The study of thymic development to a temperature change need further research, but the challenges and limitations encountered in this study will be useful in further studies. Relevant immune organs such as the kidney, intestinal spiral valve and pericardial lymphoid tissue should be studied in terms of their ontogeny and response to different external factors and stressors such as water temperature in the future. Also, the presence of maternal immune factors should be assessed in these early life stages, as they may contribute significantly for the overall immune status of these fish.

4.3. Contributions to original knowledge

- No such ontogeny studies have been previously done in sturgeon. The results obtained are an important and necessary baseline for future research on juvenile sturgeon immune responsiveness and competence.
- Rearing sturgeon at higher temperatures (up to 20°C) would be beneficial for sturgeon aquaculture, since it maximizes growth rates and positively influences immune cell percentages in relevant immune organs.
- The successful maintenance of D. oblongum parasites is a useful step in the study of host-parasite immune responses in sturgeon, since this is a commonly seen parasite in wild sturgeon.

4.4. Future research

Considering how little is known regarding sturgeon immunology, there are several areas of research worth pursuing that will help elucidate sturgeon immune responses during development and under different stimuli.

4.4.1. Maternal transfer of immune factors to its progeny

Several immune factors have been documented in fish eggs. Innate factors such as lectins[9], lysozyme[10], complement[11] and vitellogenin[12] should be measured in sturgeon eggs and in larvae after hatching until the transition to exogenous feeding. The presence of maternal transfer of specific-immune factors such as IgM[13] should also be assessed as well as when autologous IgM production is first detected in Atlantic and shortnose sturgeon eggs and larvae, since lymphocyte populations were not visible in any of the tissues studied until 768 dd in Atlantic sturgeon larvae.

4.4.2. Immune responses to different stimuli/stressors

4.4.2.1. Immunostimulant feed (β-glucan yeast-based feed): Studies done on Asian catfish (Clarias batrachus L.) regarding the effect of β-1,3 glucan on innate immunity and disease resistance[14] and Siberian sturgeon (A. baeri) on the effect of an immunostimulant feed on innate and humoral immune parameters[15] suggest that this immunostimulant might have similar beneficial effects in Atlantic/shortnose sturgeon. PCVs should be determined and innate immune parameters such as the amount of mucus secretion, mucus and serum lysozyme activities, mucus bactericidal activity, serum bactericidal activity and serum natural hemolytic complement activity should be

measured in juvenile fish. Serum IgM levels and gene expression of immune relevant genes in the spiral valve should also be assessed.

4.4.2.2. Exposure to bacterial antigens: Sturgeon can be impacted by infections caused by bacteria such as A. salmonicida[16], Flavobacterium spp and A. hydrophila[17]. A. hydrophila is a gram-negative bacteria normally part of the intestinal bacterial flora of fish, which is able to cause disease in stressed animals. In affected fish, septicemia can spread among cultured fish very quickly resulting in high mortality rates[18]. It would be useful to measure immune responses in Atlantic and shortnose sturgeon after exposure to a bacterial pathogen such as A. hydrophila. The PCV, serum lysozyme and complement levels could be determined, as well as serum and mucus bactericidal activities[15] and Ig titers against could also be determined[17].

4.4.2.3. Exposure to viral antigens: Like all higher vertebrates, fish are also susceptible to viral diseases. It would be useful to assess how Atlantic and shortnose sturgeon respond, following exposure to the synthetic dsRNA polymer poly inosinic:cytidylic acid (Poly I:C), found to stimulate IFN production in salmonids[8] and Atlantic cod[19].

4.4.2.4. Exposure to parasites: The exposure of shortnose sturgeon to sturgeon lice (D. oblongum) during the temperature trial was unsuccessful. Therefore, considering that D. oblongum is often found in wild sturgeon[20] and little is known about host-parasite interactions and host immune responses to this parasite, it would be useful to maintain the parasites in saltwater and to re-expose the fish with a higher parasite load. Considering that sturgeon tolerance to salinity increases with size[21], using 3 to 4 year old

Atlantic and shortnose sturgeon would be reasonable, since they are known to live in oceanic salinities up to 33 parts per thousand[22]. Also, a histological analysis of the skin lesions caused by the parasites could be done.

4.4.2.5. Gene expression: The limited sequences currently available for the study of the differential expression of Atlantic and shortnose sturgeon immune genes in different conditions require that novel sequences be obtained for these species. Next Generation RNA sequencing (RNA-Seq)[17] could be used to determine the transcriptional gene structure and analysis of changing gene expression levels of relevant immune genes during development and under different environmental stimuli and conditions. For both Atlantic and shortnose sturgeon, a genome transcription map would be generated de novo, which would allow for further analysis of their immune responses following exposure to pathogens (such as parasites and bacterial pathogens) and response to immunostimulants, as mentioned above.

4.5. References

1. Bronzi, P., Rosenthal, H., & Gessner, J. 2011. Global sturgeon aquaculture production: An overview. J. Appl. Ichthyol. 27, 169-175.

2. Mohler, J.W., King, M.K. & Farrell, P.R. 2000. Growth and survival of first-feeding and fingerling Atlantic sturgeon under culture conditions. N. Am. J. Aquacult. 62, 174-183.

3. Conte, F.S., Doroshov, S.I., Lutes, P.B. & Strange, E.M. 1988. Hatchery manual for white sturgeon, Acipenser transmontanus R., with application to other North American Acipenseridae. Oakland: Div. Agric. Nat. Res., University of California. Pp. 67.

4. Saurabh, S. & Sahoo, P.K. 2008. Lysozyme: an important defense molecule of fish innate immune system. Aquacult. Res. 39, 223-239.

5. Magnadottir, B., Lange, B.S., Gudmundsdottir, S., Bøgwald, J. & Dalmo, R.A. 2005. Ontogeny of humoral immune parameters in fish. Fish Shellfish Immunol. 19, 429-439.

6. Press, A. McL. & Evensen, O. 1999. The morphology of the immune system in teleost fishes. Fish Shellfish Immunol. 9, 309-318.

7. Fänge, R. 1986. Lymphoid organs in sturgeons (Acipenseridae). Vet. Immunol. Immunopathol. 12, 153-161.

8. Eaton, W.D. 1990. Anti-viral activity in four species of salmonids following exposure to poly inosinic:cytidylic acid. Dis. Aquat. Organ. 9, 193-198.

9. Kim, B., Nam, B., Kim, J., Park, H., Song, J. & Park, C. 2011. Molecular characterization and expression analysis of a fish-egg lectin in rock bream, and its response to bacterial or viral infection. Fish Shellfish Immunol. 31, 1201-1207.

10. Wang, Z. & Zhang, S. 2010. The role of lysozyme and complement in the antibacterial activity of zebrafish (Danio rerio) egg cytosol. Fish Shellfish Immunol. 29, 773-777.

11. Yousif, A.N., Albright, L.J. & Evelyn, T.P.T. 1994. In vitro evidence for the antibacterial role of lysozyme in salmonid eggs. Dis. Aquat. Organ. 19, 15-19.

12. Shi, X., Zhang, S. & Pang, O. 2006. Short communication: Vitellogenin is a novel player in defense reactions. Fish Shellfish Immunol. 20, 769-772.

13. Swain, P. Dash, S. Bal, J., Routray, P., Sahoo, P.K. Sahoo, S.K., Saurabh, S., Gupta, S.D. & Meher, P.K. 2006. Passive transfer of maternal antibodies and their existence

in eggs, larvae and fry of Indian major carp, Labeo rohita (Ham.). Fish Shellfish Immunol. 20, 519-527.

14. Kumari, J. & Sahoo, P.K. 2006. Dietary β-1,3 glucan potentiates innate immunity and disease resistance of Asian catfish, Clarias batrachus (L.). J. Fish Dis. 29, 95-101.

15. Eslamloo, K., Falahatkar, B. & Yokoyama, S. 2012. Effects of dietary bovine lactoferrin on growth, physiological performance, iron metabolism and non-specific immune responses of Siberian sturgeon Acipenser baeri. Fish Shellfish Immunol. 32, 976-985.

16. Kolman, H., Kolman, R. & Siwicki, A. 2000. Non-specific defense mechanisms of Russian sturgeon (Acipenser gueldenstaedti Brandt) reared in cages. Archives of Polish Fisheries. 8, 181-192.

17. Khoshbavar-Rostami, H.A., Soltani, M. & Hassan, H.M.D. 2007. Immune responses of great sturgeon Huso huso to Aeromonas hydrophila bacterin. J. Fish Biol. 70, 1931-1938.

18. Ardó. L., Jeney Z., Adams, A. & Jeney, G. 2010. Immune responses of resistant and sensitive common carp families following experimental challenge with Aeromonas hydrophila. Fish Shellfish Immunol. 29, 111-116.

19. Hori, T.S., Gamperl, A.K., Booman, M., Nash, G.W. & Rise, M.L. 2012. A moderate increase in ambient temperature modulates the Atlantic cod (Gadus morhua) spleen transcriptome response to intraperitoneal viral mimic injection. BMC Genomics. 13, 431.

20. Fast, M.D., Sokolowski, M.S., Dunton, K.J. & Bowser, P.R. 2009. Dichelesthium oblongum (Copepoda: Dichelesthiidae) infestation in wild-caught Atlantic sturgeon, Acipenser oxyrinchus oxyrinchus. ICES J. Mar. Sci. 66, 2141-2147.

21. Jarvis, P.L & Ballantyne, J.S. 2003. Metabolic responses to salinity acclimation in juvenile shortnose sturgeon Acipenser brevirostrum. Aquaculture. 219, 891-909.

22. Krayushkina, L.S. 2006. Considerations on evolutionary mechanisms of osmotic and ionic regulation in Acipenseridae: an overview. J. App. Ichthyol. 22 (Suppl. 1), 70-76.

23. Wang, Z., Gerstein, M. & Snyder, M. 2009. RNA-Seq: a revolutionary tool for transcriptomics. Nat. Rev. Genet. 10, 57-63.

Appendices

Appendix A

Protocol 1 - BRINE SHRIMP (Artemia franciscana) CULTURE

1. Calculate percentage of fish biomass and frequency of feeding (% bdw/day).
2. Prepare salt water (29 parts per thousand): 30g/L (NaCl in double distilled water-DDW) and confirm specific gravity with a refractometer (specific gravity should be approximately 1.022).
3. Place 1 gram of Artemia franciscana cysts in a hatching vial (6L of water).
4. Place a light above the hatching vial (Light intensity: 1000 -1050 units).
5. Place an aerator in the hatching vial to ensure oxygen supply to the cysts.
6. Maintain a constant water temperature of 25°C (25 to 30°C) by placing a heater in the vial.
7. The cysts take 24 to 48 hours to hatch in the above conditions.
8. To harvest the hatched brine shrimp, place the light under the hatching vial and wait for them to migrate towards the collecting tube in the vial before collecting (brine shrimp are drawn to light).

Protocol 2 - HEMATOXYLIN AND EOSIN STAINING PROTOCOL[14]

1. Place cut sections on glass slides.
2. Stain with hematoxylin solution
3. Wash briefly in water and differentiate in acid-alcohol.
4. Wash well in water and blue for 10 to 30 seconds. Check microscopically- the nuclei should be a deep blue color with the vesicular nuclei showing a well-marked chromatin pattern. The background should show only weak residual hematoxylin coloration.
5. Wash in water and stain with eosin solution for 5 minutes.
6. Wash quickly in water, differentiate and dehydrate in alcohol. Clear and mount as desired.

Results:

- Blue: Keratohyalin, nuclei, cytoplasmic RNA, some calcium salts, urates, bacteria (weakly).
- Bright red: Muscle, keratin, coarse elastic fibers, fibrin, fibrinoid.
- Pink: Collagen, reticulin, myelinated nerve fibers, amyloid.
- Orange: Red blood cells.

Protocol 3 - RESIN (EPON MIX)

1. Mix resin components:
    20 ml of Araldite 502
    25 ml of SPI-PON 812
    60 ml of DDSA
2. Stir mix manually with magnetic stirrer for 30 minutes.
3. Add 2.4 ml of DMP-30.

4. Stir for 30 minutes.
5. Place beaker in vacuum desiccator for 1 hour.

Calculation of resin components (see EM sample processing protocol):
I. Processing (Infiltration): The total volume of reagents per vial is 4 ml. The total resin needed for processing is 7 ml per vial:

50:50% mixture: 2 ml of resin
75:25% mixture: 3 ml of resin
100% resin: 2 ml

II. Embedding: 0.7 ml of resin per BEEM (Best Equipment for Electron Microscopy) capsule.
Total amount of resin needed for processing and embedding: 14 ml per vial.

Protocol 4 - EM SAMPLE PROCESSING

Sample fixation: Collect samples and place them in 2% glutaraldehyde (in 0.1 molar (M) sodium phosphate buffer) for 1 to 2 hours at room temperature (RT) or overnight at 4°C.

Washes: Wash samples in 0.1 M phosphate buffer for 10 minutes on a rotator. Repeat this step twice.

Post-fixation: Place samples in 1% osmium tetroxide (OsO_4) in phosphate buffer for 1 to 2 hours at RT.

Dehydration: Fresh solutions of ethanol should be used at the following sequential concentrations and the vials placed on a rotator (unless samples are delicate):

50% ethanol, 10 minutes, twice
70% ethanol, 10 minutes, twice
95% ethanol, 10 minutes, twice
100% ethanol, 15 minutes, twice
100% propylene oxide (PO), 10 minutes, twice (do not place samples on rotator)

Infiltration: Mix Epon resin with PO in a plastic beaker prior to use in the following sequential concentrations:

50-50 Epon (50% Epon: PO (50%) 30 minutes to 2 hours, on the rotator
75:25 Epon (75% Epon): PO (25%) 30 minutes to 2 hours on the rotator
100% Epon, 1 hour or overnight on the rotator or a vacuum desiccator

Embedding: place samples in labelled BEEM capsules (Better Equipment for Electron Microscopy) and fill them with 100% Epon resin.

Polymerization: polymerize samples in a vacuum oven for 24 hours at 65-70^{o}C.

Protocol 5 - THIN SECTION STAINING

1. Always float the grids on a drop of stain and place them section down.

2. Place glass plate in the hood and a parafilm square on the glass.

3. Staining sections with uranyl acetate: Place a small drop of centrifuged stain on parafilm (one drop per grid). Cover grids with a petri dish and place bibulous paper over it to protect it from the light (uranyl acetate is light sensitive). Stain grids for 30 minutes.

4. Washing: Fill three Coplin jars with distilled water. Dip the grids in each Coplin jar for 30 seconds using forceps, leave them in the forceps and cover them with bibulous paper until they dry.

5. Staining sections with lead SATO: Place pellets of sodium hydroxide (NaOH) on a fresh parafilm before adding drops of stain. Filter the lead stain, discard first few drops, place grids on top of the drop section down and stain for 2 minutes.

6. Washes: Fill three Coplin jars with fresh distilled water. Dip grids in each Coplin jar for 30 seconds using forceps, leave them in the forceps and cover them with bibulous paper until they dry.

Appendix B

Table B.1. p-values and R-Sq of meningeal myeloid tissue, spleen and thymus variables for the different age groups of A. oxyrinchus oxyrinchus. Cut-off for significance: p-value<0.05. KW: Kruskal Wallis non-parametric test. Significant differences highlighted in yellow.

Variables		Groups	R-Sq (%)
Length (cm)		<0.005	90.59
Meningeal Myeloid Tissue	Erythrocyte/ thrombocyte % (KW)	0.009	-
	Heterophil %	0.001	58.63
	Eosinophil % (ln)	0.018	48.71
	Lymphocyte % (ln)	0.120	33.49
	Reticular cell % (ln)	0.007	54.58
	Undifferentiated cell %	0.313	23.27
Spleen	Erythrocyte/ thrombocyte % (Sqrt)	0.242	23.97
	Heterophils % (Sqrt)	0.019	44.79
	Eosinophil % (ln)	0.235	24.3
	Lymphocyte % (KW)	0.018	-
	Reticular cell % (KW)	0.076	-
	Undifferentiated cell % (ln)	0.001	58.74
Thymus	Erythrocyte/ thrombocyte %	-	-
	Heterophil % (KW)	0.91	
	Eosinophil % (KW)	0.88	
	Lymphocyte % (KW)	0.331	-
	Reticular cell % (1/Sqrt)	0.233	24.14
	Undifferentiated cell % (ln)	0.018	47.90
	Mitotic cell % (KW)	0.701	-
	Necrotic cell % (KW)	0.212	-

Table B.2. p-values (Bonferroni method) of meningeal myeloid tissue, spleen and skin variables between different time points of different groups of A. brevirostrum (smaller and larger sizes) held at 11°C and 20°C. Cut-off for significance: p-value<0.05. Significant differences highlighted in yellow.

Variables		Small 11°C		Small 20°C		Large 11°C		Large 20°C	
		Between Time Points							
Mean Weight –g (ln)		1-2	>0.05	1-2	>0.05	1-2	>0.05	1-2	>0.05
		1-3	>0.05	1-3	<0.005	1-3	>0.05	1-3	>0.05
		2-3	>0.05	2-3	<0.005	2-3	>0.05	2-3	>0.05
Meningeal Myeloid Tissue	Erythrocytes/ Precursor cell % (ln)	1-2	>0.05	1-2	>0.05	1-2	>0.05	1-2	>0.05
		1-3	>0.05	1-3	0.031	1-3	>0.05	1-3	>0.05
		2-3	>0.05	2-3	>0.05	2-3	>0.05	2-3	>0.05
	Thrombocyte % (1/Sqrt)	1-2	0.133	1-2	>0.05	1-2	>0.05	1-2	>0.05
		1-3	<0.005	1-3	>0.05	1-3	<0.005	1-3	<0.005
		2-3	>0.05	2-3	0.07	2-3	<0.005	2-3	<0.005
	Myeloid progenitor cell % (Sqrt)	1-2	>0.05	1-2	>0.05	1-2	>0.05	1-2	>0.05
		1-3	0.09	1-3	>0.05	1-3	>0.05	1-3	0.154
		2-3	>0.05	2-3	0.07	2-3	>0.05	2-3	0.003
	Immature Heterophil % (Sqrt)	1-2	>0.05	1-2	>0.05	1-2	>0.05	1-2	>0.05
		1-3	>0.05	1-3	>0.05	1-3	>0.05	1-3	>0.05
		2-3	0.073	2-3	>0.05	2-3	>0.05	2-3	>0.05
	Heterophil % (ln)	1-2	>0.05	1-2	0.148	1-2	>0.05	1-2	>0.05
		1-3	0.006	1-3	>0.05	1-3	>0.05	1-3	0.004
		2-3	0.002	2-3	0.006	2-3	>0.05	2-3	0.111
	Lymphocyte % (ln)	1-2	>0.05	1-2	>0.05	1-2	>0.05	1-2	>0.05
		1-3	>0.05	1-3	>0.05	1-3	>0.05	1-3	>0.05
		2-3	>0.05	2-3	>0.05	2-3	>0.05	2-3	>0.05
	Eosinophil % (ln)	1-2	>0.05	1-2	>0.05	1-2	>0.05	1-2	>0.05
		1-3	>0.05	1-3	>0.05	1-3	>0.05	1-3	>0.05
		2-3	>0.05	2-3	>0.05	2-3	>0.05	2-3	>0.05
Spleen LM Gene Expression	White pulp % (Sqrt)	1-2	>0.05	1-2	0.43	1-2	0.36	1-2	>0.05
		1-3	0.3	1-3	0.03	1-3	>0.05	1-3	>0.05
		2-3	>0.05	2-3	>0.05	2-3	>0.05	2-3	>0.05
	IRF-1 – MNRQ (Sqrt)	1-2	>0.05	1-2	>0.05	1-2	>0.05	1-2	>0.05
		1-3	>0.05	1-3	>0.05	1-3	>0.05	1-3	>0.05
		2-3	>0.05	2-3	>0.05	2-3	>0.05	2-3	>0.05
	IRF-2 – MNRQ (ln)	1-2	>0.05	1-2	>0.05	1-2	>0.05	1-2	>0.05
		1-3	>0.05	1-3	>0.05	1-3	>0.05	1-3	>0.05
		2-3	>0.05	2-3	>0.05	2-3	>0.05	2-3	>0.05
	MMP-9 – MNRQ (ln)	1-2	>0.05	1-2	>0.05	1-2	>0.05	1-2	>0.05
		1-3	>0.05	1-3	>0.05	1-3	>0.05	1-3	>0.05
		2-3	>0.05	2-3	>0.05	2-3	>0.05	2-3	>0.05
Skin	IRF-1 – MNRQ (ln)	1-2	>0.05	1-2	>0.05	1-2	>0.05	1-2	>0.05
		1-3	>0.05	1-3	>0.05	1-3	>0.05	1-3	>0.05
		2-3	>0.05	2-3	>0.05	2-3	>0.05	2-3	>0.05
	IRF-2 – MNRQ (Sqrt)	1-2	>0.05	1-2	>0.05	1-2	>0.05	1-2	>0.05
		1-3	>0.05	1-3	>0.05	1-3	>0.05	1-3	>0.05
		2-3	0.03	2-3	>0.05	2-3	>0.05	2-3	>0.05
	MMP-9 – MNRQ (ln)	1-2	>0.05	1-2	>0.05	1-2	>0.05	1-2	>0.05
		1-3	>0.05	1-3	>0.05	1-3	>0.05	1-3	>0.05
		2-3	>0.05	2-3	>0.05	2-3	>0.05	2-3	>0.05

Table B.3. p-values (Bonferroni method) of meningeal myeloid tissue, spleen and skin variables between the different groups of A. brevirostrum (smaller and larger sizes) held at 11°C and 20°C. Cut-off for significance: p-value<0.05. Significant differences highlighted in yellow.

Variables		Time Points	Small 11°C			Small 20°C		Large 11°C
			Small 20°C	Large 11°C	Large 20°C	Large 11°C	Large 20°C	Large 20°C
Weight - g (ln)		1	>0.05	0.003	<0.005	0.028	0.003	>0.05
		2	0.27	0.001	<0.005	>0.05	0.002	0.37
		3	<0.005	0.035	<0.005	>0.05	>0.05	>0.05
Meningeal Myeloid Tissue	Erythrocytes/ Precursor cell % (ln)	1	>0.05	>0.05	>0.05	0.025	0.012	>0.05
		2	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
		3	0.09	0.21	0.017	>0.05	>0.05	>0.05
	Thrombocyte % (1/Sqrt)	1	>0.05	0.422	0.01	>0.05	0.06	>0.05
		2	<0.005	<0.005	<0.005	>0.05	>0.05	>0.05
		3	<0.005	>0.05	0.004	0.007	>0.05	0.301
	Myeloid progenitor cell % (Sqrt)	1	<0.005	>0.05	<0.005	0.002	>0.05	0.01
		2	>0.05	>0.05	>0.05	0.134	>0.05	0.33
		3	0.001	0.274	<0.005	<0.005	>0.05	<0.005
	Immature Heterophil % (Sqrt)	1	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
		2	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
		3	0.234	>0.05	0.015	>0.05	>0.05	>0.05
	Heterophil % (ln)	1	0.002	>0.05	>0.05	0.04	>0.05	>0.05
		2	>0.05	>0.05	0.008	>0.05	>0.05	0.004
		3	>0.05	>0.05	0.34	0.02	>0.05	<0.005
	Lymphocyte % (ln)	1	0.03	>0.05	0.001	0.016	>0.05	<0.005
		2	>0.05	>0.05	0.34	0.07	>0.05	0.034
		3	>0.05	>0.05	>0.05	0.05	>0.05	>0.05
	Eosinophil % (ln)	1	0.15	>0.05	>0.05	>0.05	>0.05	>0.05
		2	0.32	>0.05	0.08	0.221	>0.05	0.047
		3	0.012	>0.05	0.014	>0.05	>0.05	>0.05
Spleen LM Gene Expression	White pulp % (Sqrt)	1	<0.005	0.1	0.015	0.162	>0.05	>0.05
		2	>0.05	>0.05	>0.05	0.125	>0.05	0.184
		3	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
	IRF-1 – MNRQ (Sqrt)	1	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
		2	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
		3	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
	IRF-2 – MNRQ (ln)	1	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
		2	>0.05	>0.05	>0.05	>0.05	>0.05	0.029
		3	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
	MMP-9 – MNRQ (ln)	1	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
		2	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
		3	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
Skin	IRF-1 – MNRQ (ln)	1	0.057	>0.05	0.0026	>0.05	>0.05	0.009
		2	>0.05	>0.05	>0.05	0.04	>0.05	>0.05
		3	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
	IRF-2 – MNRQ (Sqrt)	1	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
		2	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
		3	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
	MMP-9 – MNRQ (ln)	1	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
		2	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
		3	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05

Table B.4. p-values, interactions and R-Sq (Bonferroni method) of meningeal myeloid tissue, spleen and skin variables for the different groups of A. brevirostrum (smaller and larger sizes) held at 11°C and 20°C. Cut-off for significance: p-value<0.05. Significant differences highlighted in yellow.

Variables		Groups	Time	Group*Time	R-Sq (%)
Weight - g (ln)		0.000	0.000	0.001	72.22
Meningeal Myeloid Tissue	**Erythrocytes/Precursor cell % (ln)**	0.003	0.450	0.003	41.07
	Thrombocyte % (1/Sqrt)	0.000	0.000	0.000	79.8
	Myeloid cell % (Sqrt)	0.000	0.002	0.001	73.09
	Immature Heterophil % (Sqrt)	0.062	0.659	0.026	30.58
	Heterophil % (ln)	0.000	0.000	0.038	67.36
	Lymphocyte % (ln)	0.000	0.713	0.316	54.13
	Eosinophil %(ln)	0.000	0.144	0.478	47.02
Spleen	**White Pulp % (Sqrt)**	0.000	0.081	0.001	46.90
	IRF-1 –MNRQ (Sqrt)	0.013	0.052	0.676	28.17
	IRF-2 - MNRQ (ln)	0.002	0.014	0.710	35.8
	MMP-9 – MNRQ (ln)	0.191	0.970	0.146	21.26
Skin	**IRF-1 – MNRQ (ln)**	0.000	0.029	0.137	49.41
	IRF-2 – MNRQ (Sqrt)	0.015	0.000	0.053	45.35
	MMP-9 – MNRQ (ln)	0.437	0.433	0.455	16.50

Appendix C

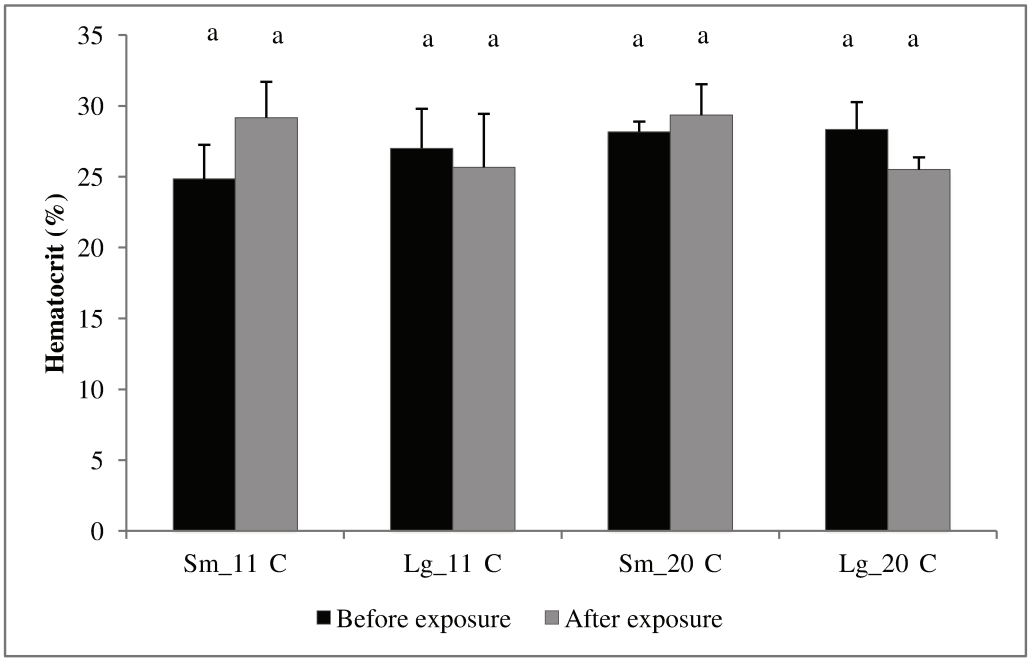

Figure C.1. Packed cell volumes (PCVs) before and after exposure to D. oblongum in different sized A. brevirostrum (smaller and larger sizes) held at 11 ^{o}C and 20 ^{o}C. n=3; different letters signify statistically significant differences between groups.

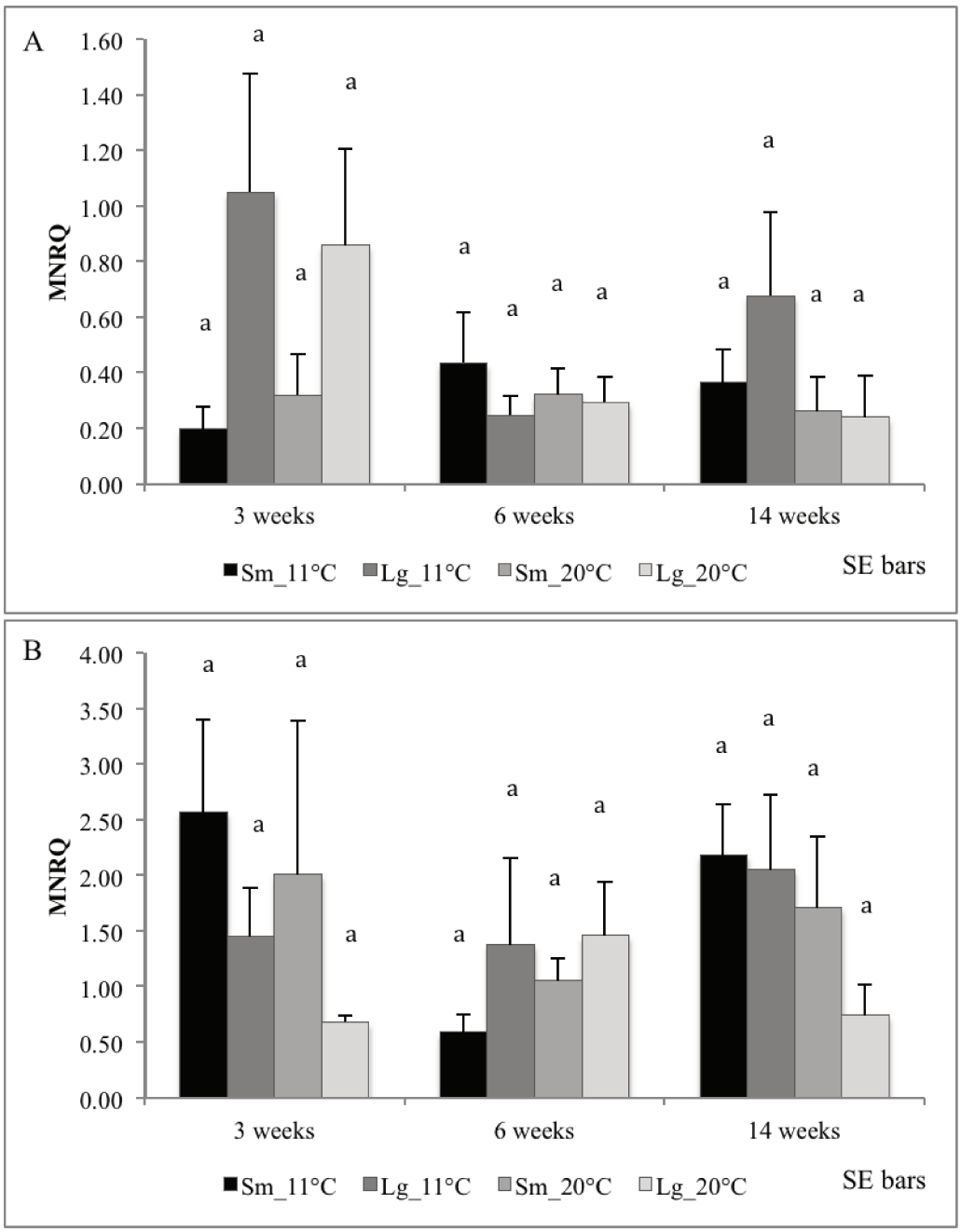

Figure C.2. MMP-9 gene expression in (A) the spleen and (B) skin in different sized A. brevirostrum (smaller and larger sizes) held at 11°C and 20°C. n=6; different letters signify statistically significant differences between groups. MNRQ (Mean Normalized Relative Quantity).